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**STUDIES ON THE ROLE OF NGF IN
ARTHRITIS-INDUCED PAIN
TRANSMISSION USING GAIT AND WEIGHT
BEARING AS OUTCOME MEASURES**

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Cover photo: Rat crossing the glass walkway in the PawPrint setup.
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Studies on the role of NGF in arthritis-induced pain transmission using gait and weight bearing as outcome measures

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To
Amilie, Johan, Anders
The meaning of my life

ABSTRACT

Pain is one of the most common reasons for seeking healthcare, with approximately forty percent of those suffering from chronic pain having joint pain. Osteoarthritis is the most common cause of joint pain, but currently there are few treatments available. The search for new, effective pain treatment has been mostly unsuccessful, in spite of the discovery of mechanisms that are involved in the transmission of nociceptive signals from the periphery to the central nervous system where pain is experienced. This work focuses on the evaluation of rodent joint pain models, the behavioural manifestations of the injuries, and the possibility to detect treatment effects in these models.

Three models have been evaluated in rats; intra-articular injection of carrageenan, Freund's complete adjuvant (CFA), and moniodoacetate (MIA) into one hind leg. In mice, two models have been evaluated; intra-articular injection of CFA, and the surgical model of anterior cruciate ligament transection (ACLT). Carrageenan injection resulted in an acute, robust inflammation, CFA injection caused a more long-lasting strong joint inflammation, and MIA injection resulted in an almost complete loss of joint cartilage after a few weeks. The model more resembling osteoarthritis was the surgical model, ACLT, which gave severe cartilage degeneration, osteophytes, and pathophysiological changes in synovia and ligaments.

Gait and weight bearing during locomotion have been tested in all models. The degree of weight bearing reduction in the affected limb was largest in the CFA- and carrageenan-induced model, followed by the MIA model and least effect was seen in the ACLT surgical model. Thus the ACLT model was not possible to use for pharmacological evaluation of drugs, whereas carrageenan- and CFA-induced monoarthritis resulted in a big enough difference between animals with monoarthritis and those without, to test drugs commonly used for pain as well as those under investigation for effects on pain.

Conventional pain relieving drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) and opioids were able to normalize effects on weight bearing caused by both the carrageenan- and the CFA-induced monoarthritis, as were treatments based on inhibiting the NGF-TrkA pathway; an anti-NGF antibody and two pan-Trk compounds. However, an antagonist of the TRPV1 receptor lacked effect.

We also investigated mice with a mutation in the R100 NGF β gene (hR100E), in comparison with mice possessing a human wild-type NGF (hWT), similar but not exactly like the one found in a hereditary sensory and autonomic neuropathy type V (HSAN V) disorder. This disorder leads to insensitivity to deep pain in homozygous patients, with sensory and autonomic functions remaining almost normal. In mice with the hR100E mutation, we found similar behavioural outcome; normal peripheral sensory functions but less pain-like behaviour when assessing joint pain with gait and weight bearing.

In summary, this work shows that in order to detect translatable effects on joint pain, models need to be robust enough, especially for pharmacological testing, but more important, the methods of testing need to be relevant for the study aim.

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Manuscript

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LIST OF ABBREVIATIONS

ACLT	anterior cruciate ligament transection
CI	confidence interval
COX	cyclooxygenase
CNS	central nervous system
DWB	Dynamic Weight Bearing
ECL	electro-chemiluminescence
FCA/CFA	Freund's Complete Adjuvant
HBC	hydroxypropyl- β -cyclodextrin
HPMC	hydroxypropylmethylcellulose
HSAN	hereditary sensory and autonomic neuropathy
IL	interleukin
LOQ	lower limit of quantification
MCC/NaCMC	microcrystalline cellulose/sodiumcarboxymethyl cellulose
MCP-1	monocyte chemoattractant protein-1
MIA	monosodium iodoacetate
MIP-1	macrophage inflammatory protein-1
NGF	nerve growth factor
NK-1	neurokinin-1
NSAID	non-steroid anti-inflammatory drug
OA	osteoarthritis
OARSI	Osteoarthritis Research Society International
PGE2	prostaglandin E2
PVP	polyvinylpyrrolidone
RA	rheumatoid arthritis
SDS	sodiumlaurylsulphate
SEM	standard error of the mean
TNF	tumor necrosis factor
Trk	tropomyosin receptor kinase
TRPV1	transient receptor potential vanilloid 1
UV	ultraviolet

1 INTRODUCTION

1.1 BACKGROUND

Chronic pain is a major health problem affecting 10-20 percent of the population, resulting in marked reduced quality of life for the individual. Pain is also a substantial socio-economical problem and the cost for chronic pain in Sweden has been estimated to 87.5 billion SEK in medical treatment, loss of productivity and disability (SBU 2006). A large-scale survey undertaken in 15 European countries showed that 40% of the people reporting persistent pain (> 6 months) had joint pain. OA, followed by RA, is the most common cause of joint pain (Breivik et al., 2006). In these conditions, disability and pain upon movement are frequently reported to have a significant impact on the quality of life (Rejeski et al., 1996; van Baar et al., 1998).

Thus the overall aim of my work has been to elucidate the possibility to use measurement of weight bearing and gait in rodent models of joint pain, in hopes that it would give results that are more predictive for the clinical outcome in trials with patients experiencing joint pain. As my interest and motivation is based on many years of looking at animal behaviour, that is where my focus lies. So far several new chemical entities have been tested in the clinic based on other measurements pre-clinically, such as effects on mechanical or thermal sensitivities, and as these attempts have not proven successful my conviction is that new ways of measurements are needed. Drug development in the area of chronic pain has hitherto been mostly unsuccessful, and there are still few available effective treatments for chronic pain conditions. This is in spite of immense resources being spent on developing new molecular targets and testing them, first thoroughly in the preclinical setting, and later some selected promising molecules in the clinic. Several potential new drug targets have been tested in patients, and despite positive outcomes in preclinical models, not many have been progressed to produce drugs that alleviate pain in humans. Although the majority of the preclinical targets never reach the stage of testing in humans, even those that do mostly failed. This is not a problem only for the pain indication but several of the large pharmaceutical companies, including AstraZeneca, have closed down their research in the central nervous system (CNS)/pain field and with that all research for new pain medication. As I have personal experience of working in projects bringing chemical compounds all the way to clinical testing, I feel deeply that whatever my experience could add to the possibility of finding new treatment for chronic pain would make my life's work worthwhile.

When I started looking at joint pain models more than twenty-five years ago, not many of the models and methods used today were known. Manual scoring to assess guarding behaviour had recently been published by Coderre and Wall 1987, and that was what I used in some models of monoarthritis. However the results were not satisfactory for me as I could see that scores changed over the 4 minute films that I analysed, and there were no suggestions of how to adjust

the scores for these temporal changes. New devices to measure static weight bearing encouraged me to look also for the possibility to measure as the animal walked, and in collaboration with Frank Hamers who developed the CatWalk, the analysis algorithms were improved by adding light intensity as an indirect method to measure weight bearing. By using this way of assessing the effect of inhibitors of the nerve growth factor (NGF) pathway – which worked better than any treatment I had hitherto tested – and of the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor – which had no effect – my belief in choosing a model, and even more importantly a mode of measuring effects, that mirrors what patients complain about grew stronger.

As OA is the most common reason for joint pain, and no good disease modifying medication exists today, these patients have been chosen for many of the clinical trials. Below I will review what is known about risks for getting OA, as well as OA pathology and the treatments used for OA pain today. Then follows a part where possible mechanisms of pain in OA are discussed. The next part describes animal models of joint pain; the ones I have used and some other possible models, and different ways of measuring outcome in the animal models. Finally a part on pharmacology, including possible targets for treatment that I have used in my studies.

1.2 OSTEOARTHRITIS

1.2.1 Risks for getting osteoarthritis

What causes the start of processes in the joint that lead to OA is not yet known, but several underlying risks for developing OA have been found (Felson et al., 2000; Zhang et al., 2010). As in many other joint diseases there is a difference of OA prevalence between women and men; females generally have a higher risk at getting OA and it gets more severe compared to that in males (Srikanth et al., 2005; Glass et al., 2014). This becomes more obvious as patients grow older, and age is a high risk in itself (Zhang et al., 2010; Neogi and Zhang 2013; Loeser 2013), and perhaps one of the strongest risk factors for OA (Lawrence et al., 2008). Furthermore fractures of the meniscus and injuries of the cruciate ligaments surrounding the knee increase the risk of later getting OA (Englund et al., 2003; von Porat et al., 2003; Muthuri et al., 2011), but damage to the meniscus and disruption of the anterior cruciate ligament have also been found in OA patients without previous injuries (Hill et al., 2005; Englund et al., 2003), and could perhaps be another part of the pathology itself. Another factor shown to have an impact on the occurrence of OA is body weight, and obesity adds to the development of OA (Felson et al., 1988; Niu et al., 2009; Blagojevic et al., 2010). The reason for the increased risk may be the obvious increased load on joints, but there are also suggestions that the reason is metabolic, as joints that are not loaded such as the hands and shoulders are more affected in patients with obesity (Oliviera et al., 1999). In addition, there is a large and complex genetic component to the risk of developing OA, and it has been reported that the heritability of OA is more than 60%, but differs with respect to the different joints (Spector and MacGregor 2004).

1.2.2 Osteoarthritis pathology

Osteoarthritis is a disease involving structures of the entire joint.

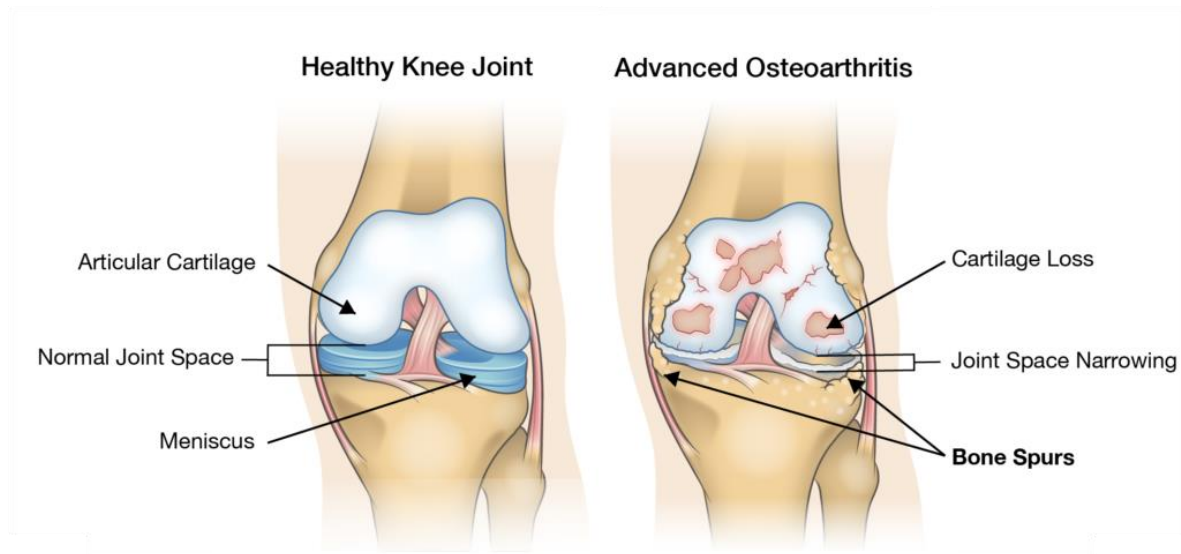


Figure 1. A healthy knee joint (left) and a knee joint with advanced osteoarthritis showing cartilage loss, joint narrowing and bone spurs. (With permission from Spring Loaded Technology. <https://springloadedtechnology.com/guide-to-severe-knee-osteoarthritis-treatment-options/>)

Destruction of the cartilage was long believed to be the major cause of OA, but inflammation of the synovium and synovial membrane, development of osteophytes (“bone spurs”) and breakdown of subchondral bone including bone marrow oedema and lesions, “characterized by excessive water signals in the marrow space on magnetic resonance imaging or ultrasound” according to Eriksen 2015 has also been suggested. The cartilage which in healthy subjects serves as a smooth lubricated surface to allow for efficient motion letting the different parts of a joint move easily one against the other is where changes are first seen. As reviewed in Loeser et al., 2012, the cartilage matrix chondrocytes undergo changes that lead to cell proliferation and cluster formation. These cells increase the production of matrix proteins and matrix-degrading enzymes, including aggrecanases and collagenases, which lead to e.g. matrix remodelling. Loeser et al., 2012 state that “once the collagen network is degraded, it appears that a state is reached that cannot be reversed”. The clustered chondrocytes, especially, have receptors for components of the extracellular matrix and cytokines and chemokines, and when activated by those modulate inflammatory and catabolic responses (Loeser et al., 2012).

A low grade inflammation of the synovium has been found even in the early stages of the disease in OA patients, and increased as the OA progresses (Benito et al., 2005; Krasnokutsky et al., 2011). In addition, macrophages and lymphocytes have been detected in the synovium of over 50% of patients with knee OA (Pearle et al., 2007). There are a number of cytokines and chemokines released from these inflammatory cells, including but not restricted to interleukin-1 (IL-1), IL-6, IL-8, tumour necrosis factor (TNF), monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 (MIP-1). But, the levels of those proteins in OA synovial fluid are not as high as in patients with RA.

Both mechanical load and the inflammatory process of OA leads to adaptations of the bone, making it less resistant to damage. Bone remodelling is thought to represent attempts to heal the bone, and may be the starting point of osteophytes, i.e. bony protrusions at the edge of the joint. As a response to the injury, including bone marrow lesions, the tissue is activated and new vascular channels grow into the affected bone to start the regeneration (Loeser et al., 2012). Along the vessels sensory nerves may grow, that are believed to add to the pain signalling in OA (Suri et al., 2007).

1.2.3 Current treatments for osteoarthritis

Treatments available today for OA do not stop disease progression, but are aimed to alleviate the major symptom, pain (Jordan et al., 2003; Zhang et al., 2007; Zhang et al., 2008; Bruyère et al., 2014). The only “treatment” with proven efficacy is physical exercise, and both aerobic training and strength exercise give the largest effects in relieving pain and improving function (Walker-Bone et al., 2000; Bennell and Hinman 2011; Juhl et al., 2014). In addition, for patients with obesity, body weight reduction is important and will relieve symptoms. However, both exercise and weight reduction necessitates behavioural changes that are difficult to control and are challenging for patients as positive results are not immediate, and so the most common treatment remains pharmacological. This is mainly pain reducing medication such as paracetamol, non-steroidal anti-inflammatory drugs (NSAIDs) and weak opioids (Felson 2009). But, according to a review by Smith et al., 2016, these treatments are similar in their efficacy but can only reduce the pain with about 20%. As the condition proceeds and OA reaches a stage with severe pain, excessive degeneration of the joint and increasing levels of disability, many go through a total joint replacement. Unfortunately as many as 15%-30% of those patients still report considerable pain (Hawker 2006; Wylde et al., 2011). As OA is a chronic condition, pain medication is needed for many years. The long-term pharmacological treatments recommended today come with considerable complications; gastrointestinal, cardiovascular and renal adverse side effects for NSAIDs and nausea, constipation and dizziness for opioids (Avouac et al., 2007; O’Niel et al., 2012; Pelletier et al., 2016). When considering this, it is obvious that there is a great need for better medication.

Drug development in the area of chronic pain has been insufficient in general, and there are currently few available effective treatments. Thus, it is critical to increase our understanding for how chronic pain is regulated in order to identify new biological targets. Several potential new drug targets have been tested in patients, and despite positive outcomes in preclinical models, very few have been progressed to produce drugs to alleviate pain in humans. Hence, it is critical to improve our tools for assessing potential new targets in a way so that they predict clinical efficacy.

1.3 PAIN MECHANISMS

In the joint, the ligaments, fibrous capsule, meniscus, periosteum and synovial layer are innervated by sensory A β , A δ and C-fibres and noxious (pain) sensation can be evoked from these structures (Schaible et al., 2009). However the cartilage is not innervated and it is believed that no sensation can be emitted from cartilage in healthy subjects. Upon activation of the sensory nerves, signals are sent to the spinal cord dorsal horn where these primary afferents couple to second order neurons or interneurons, which project the information towards the brain. Patients with joint inflammation experience pain during normal movement of the joint and even gentle pressure, e.g. palpation, often elicits pain. The increase in sensitivity is thought partly to depend on inflammatory factors released locally in the joint. For example prostaglandins, bradykinin, growth factors such as NGF, tumour necrosis factor and interleukins reduce the threshold for activation and increase the responsiveness of the sensory neuron, giving rise to pain sensations in response to both noxious and non-noxious stimuli (termed hyperalgesia and allodynia, respectively) (McMahon et al., 2005).

There is mounting evidence that there is a comparable release of similar mediators in the spinal dorsal horn, where these factors mediate spinal facilitation of pain processing, further amplifying nociceptive signals conveyed to the brain. Hence, many factors that are involved in the inflammatory process at the peripheral site may also drive spinal sensitization. It is important to note that an inflammatory response normally is self-limiting, serving as a protective mechanism in response to injury or infection. All the same, there are many long-term or chronic conditions in which inflammation does not resolve, and this may have a significant impact on the sensory neurons and lead to chronic pain.

Lately many studies have shown that sensitization of the central pain system occurs which contributes to pain in preclinical models of OA (Abaej et al., 2016; Knazovicky et al., 2016), and in chronic OA pain patients (Akinci et al., 2016; Moss et al., 2016). Both central integration of the pain signal and the descending pain pathways may play a part in the sensitization (Arendt-Nielsen et al., 2015; Sikander et al., 2016). The signs include increased spread of tenderness in areas not associated with the affected joints, shown e.g. by decreased thresholds for mechanical sensitivity. Additionally, there are reports that OA with time can manifest in neuropathic pain states (Dimitroulas et al., 2014; Roubille et al., 2014).

1.4 ANIMAL MODELS OF INFLAMMATORY PAIN AND OSTEOARTHRITIS

In order to study chronic inflammatory joint pain, the choice of animal model is important. The optimal model would be one that completely mirrors what is found in human patients, but as even in those there is a range of different structural and sensory changes, this becomes nigh impossible. Several aspects then form the basis for which model to choose. If the main focus is to look at structural changes the model needs to be one showing similar modifications, e.g. in cartilage, synovium and subchondral bone, as those seen in at least a subgroup of patients.

As my interest lies in measurement of pain-like behaviour, the models chosen for my work need to give significant effects in tests aimed at assessing mechanical or thermal sensitivity, or even more important, in tests attempting to measure spontaneous or non-evoked pain. Several widely used animal models of arthritis-like pain involve intra-articular ankle or knee injection of chemical agents such as carrageenan and Freund's complete adjuvant (FCA/CFA).

1.4.1 Carrageenan

The carrageenan-induced monoarthritis produces a pronounced inflammatory reaction where granulocytes rapidly infiltrate the site of injection and which gives nociceptive behaviour in rats that lasts for 24-48 hours. It contains a sulphated mucopolysaccharide derived from *Chondrus crispus* or from red Scottish seaweed (Smith and Cook 1953) and a single intra-articular injection can reduce the proteoglycan content and the rate of proteoglycan synthesis in the cartilage (Lowther and Gillard 1976; Santer et al., 1983). In 1992, Tonussi and Ferreira described how carrageenan injected into the rat knee joint caused a concentration-dependent incapacitation which reached a maximum within 24-48 hours after the injection. In addition, it caused a pronounced swelling of the joint. As there is a possibility that the swelling itself could cause sensory activation, dextran was injected intra-articularly into the knee joint in another group of rats. However dextran, a substance that causes oedema but does not involve inflammatory mediators or cellular infiltration, failed to induce any incapacitation.

1.4.2 FCA

FCA/CFA is a mixture of mineral oil and inactivated, dried mycobacteria, which produces a more prolonged stimulation of cell-mediated immune responses leading to a local joint inflammation that lasts for up to two weeks or more in rats. In 1992 Butler et al. described a method to induce an inflammation locally in the ankle joint of one hind paw in rats. This new technique of intra-articular injection led to a restricted monoarthritis, not the generalized polyarthritis which is a result of injecting FCA subcutaneously (Waksman et al., 1960; Pearson et al., 1960; Pearson 1963). In contrast to the carrageenan model, injection of FCA to the rat ankle joint leads to a number of structural changes such as pannus formation, bone remodelling and cartilage erosion (Brenner et al., 2006; Hashmi et al., 2010).

1.4.3 MIA

A low-grade inflammatory model, first described by Kalbhen DA in 1987: "Chemical model of osteoarthritis – a pharmacological evaluation" is induced by the intra-articular injection of monosodium iodoacetate (MIA) that inhibits glyceraldehyde-3-phosphate dehydrogenase and disrupts the chondrocyte glycolysis leading to ensuing cell death. In the MIA model the major inflammatory phase resolves within a week, while collagen degradation progressively makes the cartilage thinner until the underlying bone emerges (Guzman et al., 2003; Fernihough et al., 2004).

These three rat models are commonly used in the pain research field and have the advantage of a predictable onset, high success rate and fast disease progression. They are also the models that form the basis for my work. However, it has been debated whether using longer-lasting

animal models would generate changes in the sensory system that more closely mimic changes in the mechanisms of chronic arthritis-induced pain in humans, and efforts have been made to find models that more resembles osteoarthritis.

1.4.4 Surgical OA models

One of the most common ways of modelling OA to understand the histological consequences is by inflicting a surgical injury to the knee joint in different animal species. Several procedures have been described, such as meniscal tear, partial or total meniscectomy and anterior cruciate or other ligament transection, and all could be assumed to model aspects of traumatic causes of OA in humans. By inflicting destabilization of the knee joint in rats or mice, similar structural changes to those in patients have been described, such as loss of cartilage, occurrence of osteophytes and subchondral sclerosis (Fernihough et al., 2004; Janusz et al., 2002; Bove et al., 2006). In addition, several of these models show development of mechanical hypersensitivity and guarding of the injured limb. When comparing to the slowly evolving human OA, these models have the advantage of a slower induction (weeks) and a longer lasting (months) hypersensitivity than those chemically induced. Although differing in the pathology, surgical models could potentially be used to evaluate new mechanisms and targets for treatment of joint pain.

1.4.5 Joint loading model

Recently, a mouse model focusing on increased loading of the knee joint was developed and validated (De Souza et al., 2005; Poulet et al 2011, ter Heegde et al 2019). This model was induced by putting one hind leg of an anaesthetized mouse in a holder, where the knee joint was placed into one cup and the ankle joint into another. A small baseline load, 2N, kept the leg in place, and the load was then increased to 9-11N for 0.05 seconds every 10 seconds. The peak loading was repeated 40 times in one session, and three sessions per week was performed for two consecutive weeks. The model results in cartilage lesions and loss of structural integrity of the lateral femur (Poulet et al 2011), as well as development of mechanical hypersensitivity and reduced weight bearing of the affected hind limb, lasting for more than six weeks (ter Heegde et al 2019).

1.4.6 Spontaneous models

Spontaneously occurring knee OA has been observed in several animal species, including some strains of mice, guinea pigs and hamsters (Walton 1979; Nordling et al., 1992; Silverstein and Sokoloff 1958; Bendele and Hulman 1988). Even though one may think that these animals would be the best in which to study OA, it is impossible to know exactly when symptoms appear in the individual mouse or guinea pig. As a result, planning studies are difficult and the time to conduct a study can become far longer than resources allow for. In addition, the pathogenesis of this OA is not completely understood (Bendele 2001). These facts make well powered, planned and controlled tests expensive and challenging, if not almost impossible.

1.5 ASSESSMENT OF PAIN-LIKE BEHAVIOUR IN RODENTS

Assessment of pain-like behaviour in rodents remains a challenge, more so in mice than in rats. Behavioural testing in rodent pain models can be divided into two categories: performer stimulus-evoked, and non-evoked or spontaneous behavioural responses. The traditional way to test is by establishing a threshold where the animal reacts with a behaviour that can be assumed to be a response to a nociceptive signal – an evoked response to mechanical, thermal or cold stimuli.

1.5.1 Sensory testing

1.5.1.1 *Mechanical sensitivity*

As far back as 1896 Max von Frey in his dissertation described the use of human and horse hairs of different stiffness for detecting mechanical sensation, and since then similar artificial hairs have been named von Frey filaments (von Frey 1896). The traditional material used for the test filaments is synthetic fibres such as nylon, but as those are sensitive to changing temperature and humidity (Andrews 1993; Ängeby Möller et al., 1998) new hairs made of glass fibre, the von Frey Optihairs, have also been introduced (Fruhstorfer et al., 2001). Several different approaches have been used to establish a threshold: application of one or more selected filaments for a specified number of times and counting percentage of responses to each filament; application of filaments with increasing force to find the one where the animal starts responding; or using the up-down technique first described by Dixon in 1980, and modified by Chaplan et al. 1994. Whichever method of testing that is chosen, they are all dependent on a performer and his or her subjective decision of when a withdrawal response is achieved. To overcome the subjectivity attempts have been made to develop more objective methods, using an electronic von Frey filament which is applied to the paw with increasing force and that gives a threshold automatically as the paw is withdrawn (Ängeby Möller et al., 1998; Cairns et al., 2002). For testing sensitivity to pressure in deeper lying tissues several devices have been presented, such as that described by Randall and Selitto (Randall and Selitto 1957).

In my work before becoming a PhD student I have tested mechanical sensitivity in rats with both traditional nylon von Frey filaments (Stoelting) and von Frey Optihair, as well as an electronic von Frey device developed by Somedic (Ängeby Möller et al., 1998). The up-down method to calculate a withdrawal threshold was always used, as this method seems to have become the method of choice for many pain researchers. However the majority of these studies were performed on rats subjected to models of neuropathic pain. In my present work the more stable von Frey Optihairs have been used.

1.5.1.2 *Thermal sensitivity*

Testing sensitivity to heat comes with similar difficulties. Thus directing a light beam towards the plantar skin of a paw to assess the latency until withdrawal was described by Hargreaves 1988 (Hargreaves et al., 1988), but as the skin can have varying basic temperature which affects the result it can be difficult to establish what has more impact – the increase in temperature or the starting skin temperature (Tjølsen et al., 1989). When the baseline skin temperature is high,

the latency until withdrawal is short, while it takes considerably longer time until withdrawal if the skin temperature in the beginning of testing is low (Hole and Tjølsen 1992).

1.5.1.3 Cold sensitivity

Cold sensitivity is often measured with the application of an acetone drop (De la Calle et al., 2002; Dowdall et al., 2005), but spraying of ethyl chloride has also been reported (Gustafsson and Sandin 2009). These applications are considered to be pain-like if the animal starts licking and/or shaking the affected paw. The decrease in skin temperature following application is much larger for ethyl chloride, but in both cases the amount applied is hard to control as is the exact site of stimulation.

1.5.2 Assessment of non-evoked or spontaneous behaviours

Still, for models of arthritis it is also possible to assess spontaneous or non-evoked behavioural responses, which probably more reflect the sense of pain in the animals. This has been the main focus for my work, as I believe that this could mirror what patients experience in the clinic. I have specifically tried to find ways of measuring pain-like behaviour of animals in locomotion, as that is the major complaint in patients with joint pain. Many ways of measuring total locomotor activity exist, but there are equally many reasons for a decrease in locomotor activity, making this way of testing specific models difficult.

1.5.2.1 Static weight bearing

When only one hind paw has been subjected to an induction of arthritis, scoring of guarding behaviour during standing and walking was first described by Coderre and Wall (Coderre and Wall 1987). Scoring is by its nature subjective and many are those that have come up with methods trying to make assessment of guarding more objective. By fitting force plates in the floor of a box the weight load of the two hind paws could be measured (Schött et al., 1994), and later the Incapacitance tester was introduced where animals stand with the two hind paws on force plates while the front paws are placed on a ramp (Bove et al., 2003). In both methods, placing an animal in the box or holder induces stress and needs training before a trustworthy result can be obtained, and varying placements of the tail or the animal leaning onto the side of the box can distort the readout.

1.5.2.2 Stationary weight bearing

Recently another device has been introduced, the Advanced Dynamic Weight Bearing (DWB), where a sensor mat measures the load and a camera fitted on top of the box films the animal simultaneously making it possible to validate the detection of each paw's placement (Tetreault et al., 2011). The animal is allowed to move within a restricted area during a selected period to give weight bearing data of all four paws both when standing and when rearing.

1.5.2.3 Gait and weight bearing during locomotion

As movement-induced pain is a major complaint in patients with chronic pain from the joints, exploring behaviour in rodents with and without joint inflammatory pain during locomotion

may give added information of mechanisms and treatment. Several ways to do this have been introduced, but few have yet been extensively used.

Tonussi and Ferreira (Tonussi and Ferreira 1992) invented a setup where rats were fitted with specially designed metal gaiters wrapped around the paw of interest, and the time of contact with a mesh floor covering a rotating cylinder was then assessed. With this device they could establish the pharmacological effects of some analgesic drugs after injection of carrageenan into one knee joint of rats. By attaching an electrode between the pads of the hind paw the method was improved and the rats could walk more freely on the cylinder (López-Muñoz et al., 1993). Still these were setups that were complicated and difficult to manufacture to become commercially available.

Another approach was introduced – to use video recordings from underneath to assess walking rats. To make the paw contact areas more visible a frosted plexiglass surface was suggested, which gave several new parameters of all four paws such as gait stance – the time from initial contact of a paw with the floor until lifting the paw as next step was started (Walker et al., 1994). The weight bearing during locomotion turned out to be more difficult to assess. By adapting a technique described by Betts and Duckworth in 1978 for measuring human plantar pressure, based on white light entering the long edges of a 6 mm thick glass where the light was internally reflected between the two surfaces of the glass until e.g. a rat paw was placed which then scattered the light, K Clarke built a walkway that could study the paw contact patterns of rats (Clarke 1992). He later combined it with load measurements underneath two 10x5 cm areas at half-way of the walkway, one for each side of the glass floor, and showed that the coefficient of correlation between load cell output and output from the scattered light of a paw in contact with the floor was 0.92 (Clarke 1995). Using this setup, effects of the MIA model of OA in rats, as well as detailed load and temporal aspects of mouse locomotion were assessed (Clarke et al., 1997; Clarke and Still 1999; Clarke and Still 2001).

Hamers et al., 2001 introduced a walkway with light entering the long edge of a glass floor, the CatWalk, where a semi-automated interactive analysis algorithm gave a large number of parameters assessed in rats with spinal cord injury (step sequence distribution, regularity index, print area, maximum contact area during stance, base of support, stride length, swing duration, and duration of tail dragging and abdominal dragging). Later the amount of light intensity per pixel was added to make it possible also to indirectly assess weight bearing in rats with a model of neuropathy (Vrinten and Hamers 2003). Similar setups have followed, manufactured by several companies focusing on devices for animal behavioural research.

Others tried a slightly different technique, such as that of the TreadScan (CleverSys) where rodents' paws are filmed from underneath as they walk on a sliding treadmill belt which can be controlled for the speed of the animal as it is forced to move at the same speed as the belt (Beare et al., 2009). Temporal and all area-dependent parameters can be assessed, and it's

suggested that changes in weight bearing are indirectly detected as the paw is pressed against the floor and the colour of the plantar skin becomes lighter when more of the small capillaries are obstructed.

Still, the most common tests rely on tester-evoked responses such as assessment of mechanical or thermal hypersensitivity by generating a withdrawal from the source of stimulus. According to Mogil and Cragger 2004, 90% out of 259 articles between 2000 and 2004 in the journal “Pain” that described animal studies of neuropathic and inflammatory pain in which behavioural testing was included, exclusively measured changes in threshold or response to evoked stimuli. A number of drugs, for example neurokinin-1 (NK-1) antagonists and transient receptor potential vanilloid 1 (TRPV1) antagonists (Svensson et al., 2010) have failed in clinical trials despite repeatedly showing effect against hypersensitivity states. There is an increasing suspicion that tester-evoked tests do not reflect the human joint pain problem, and that non-evoked behaviours such as locomotion, guarding of the effected limb, reduced weight bearing and gait changes may provide a better correlation to the human pain state.

Hence, it is critical to improve our tools for assessing potential new targets in a way so that they predict clinical efficacy. From a pain perspective, it is essential to evaluate models that best mimic the human pain condition, and perhaps even more important, which endpoints to use when measuring pain behaviour. As movement-induced pain is a problem for arthritis patients we have investigated if gait and weight bearing can be used as endpoints that better mimic the pain state in humans, and thus hopefully better predict results in clinical trials.

1.6 PHARMACOLOGY

Pharmacological evaluation was performed in the present work, to elucidate the usefulness of the chosen models and measurement methods, and include several NSAIDs, paracetamol, pregabalin and opioids. In addition new chemical entities targeting the NGF pathway – both an anti-NGF antibody and two pan-Trk inhibitors; inhibitors of tropomyosin receptor kinases (Trk) A, B and C – and an inhibitor of the TRPV1 receptor have been evaluated. Thus, below I describe some of the characteristics of these pharmacological agents.

1.6.1 Drugs inhibiting NGF and its receptor TrkA

Several attempts have been made to find suitable pharmacological treatment for chronic pain using the NGF/TrkA pathway as target. One of the most encouraging is the development by Pfizer of a monoclonal antibody that inhibits NGF, Tanezumab, which shows efficacy both in rodent models (Shelton et al., 2005) and in patients with OA (Lane et al., 2010). The reduction of knee pain in the patients while walking was more pronounced than that being achieved by commonly used NSAIDs (Schnitzer et al., 2015). NGF is a member of the neurotrophin family and activates the TrkA with high affinity, and the p75 receptor with low affinity. It is essential for axonal growth and survival, especially in the neonatal time period, and later in life plays a major role in pain processing. When tissue is injured or inflamed, NGF is released from mast

cells, macrophages, keratinocytes and T-cells (Figure 2; for review see Pezet and McMahon 2006). Of note, when activated, the TrkA receptor forms a complex with NGF and is internalized and transported to the nucleus where it promotes expression of a number of other pain-associated receptors, such as CGRP, TRPV1, P2X3 and ion channels neurotransmitters (Heppenstall and Lewin 2000; Winston et al., 2001; Bonnington and McNaughton 2003; Pezet and McMahon 2006).

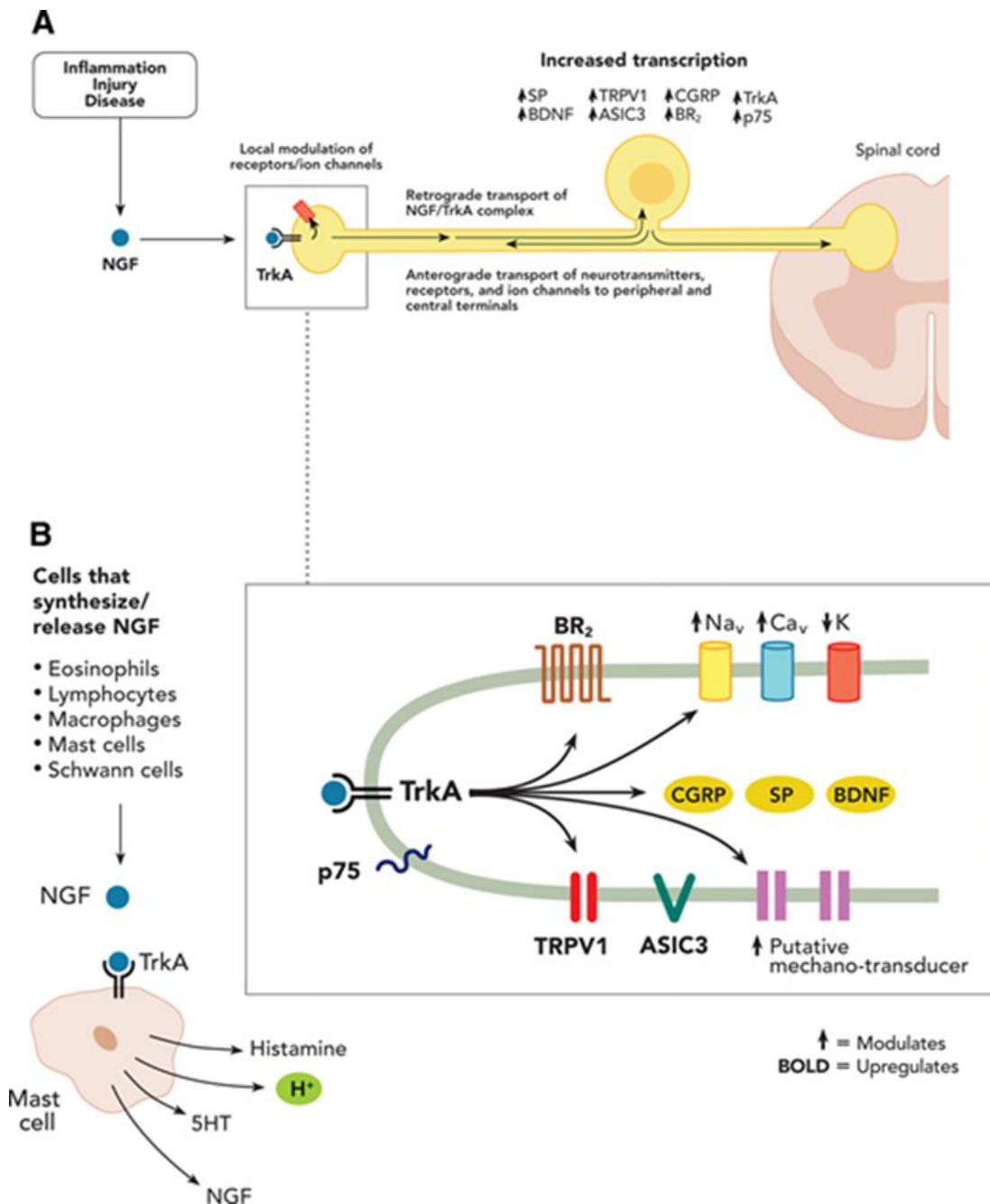


Figure 2. Neurotransmitters, receptors and ion channels that are modulated and up-regulated by NGF binding to TrkA-positive primary afferent sensory nerve fibres are shown in (A). In (B), cells that release NGF during injury or inflammation is shown. (Reprinted with permission from Mantyh et al., 2011)

Injection of NGF into the paw causes hypersensitivity to mechanical stimuli in rats (Lewin and

Mendell 1993), as does injection into the masseter muscle in humans, where pain intensity also increased when chewing and yawning (Svensson et al., 2003). NGF is elevated in synovial fluid from patients with RA (Halliday et al., 1998), however the effect of NGF blockage on pain in RA animal models or RA patients has not been extensively studied.

1.6.2 Inhibitor of the TRPV1 receptor

The TRPV1 receptor is activated by heat and acidic environment containing increased levels of H⁺ (Caterina et al., 1997). Capsaicin, the ingredient giving food some of its “hot” taste, is an agonist of TRPV1, and injecting capsaicin into human or animal skin results in an intense but short-lasting pain (Lamotte et al., 1992; Kinnman and Levine 1995). Activation of the TrkA receptor by NGF leads to a rapid increase in TRPV1 receptor membrane expression on primary sensory neurons (Zhang et al., 2005; Stein et al., 2006) and selective antagonism of the TRPV1 receptor has been suggested as a potential drug target for treatment of joint inflammation (Keeble et al., 2005). A model of heat pain that is commonly used in clinical trials is the ultraviolet (UV) burn, and TRPV1 antagonists have been shown to alleviate this pain. However trials with TRPV1 antagonists in patients with OA failed as no effect on pain was found (Svensson et al., 2010; Segerdahl et al., 2010).

1.6.3 Non-steroidal anti-inflammatory drugs

One of the mediators produced in inflamed tissue is prostaglandin E₂ (PGE₂). The origin is fatty acids derived from cell membrane, and it is synthesized when the tissue is injured by physical trauma or when cytokines or growth factors such as NGF signal the need for tissue inflammation and repair. The production of prostaglandins requires several enzymes, one of which is cyclooxygenase (COX), the enzyme inhibited by NSAIDs. There are two different variants of COX; COX-1 and COX-2. COX-1 is believed to be constitutive, whereas COX-2 is predominantly produced in inflammation. Production of PGE₂ leads to the well-known symptoms of inflammation – pain, swelling and fever.

The first report of the effects of COX inhibition in humans through use of willow bark was found in: Stone E. “An account of the success of the bark of the willow in the cure of agues” (Stone 1763), and reported in Vane and Botting 1998. However willow bark, and other plant derived decoctions containing COX inhibiting salicylates have been used for thousands of years, and are known from the ancient Egyptians through the Ebers papyrus, and ancient Greeks in recommendations by Hippocrates. Still today, COX inhibitors are the most commonly used pain relieving drugs and are recommended for many conditions that have an inflammatory component, including joint pain originating from e.g. OA.

1.6.4 Opioids

Inhibition of the opioid receptor has been used to relieve pain for thousands of years as well, but not until the last century have there been an opportunity to reveal the mechanism behind the effect. Today we know that opioids preferentially act on the μ opioid receptor subtype, a G-protein coupled receptor which has effects on ion-channels in the membrane of nervous tissues, resulting in a reduction of neuronal excitability and transmitter release (for review see Corbett et al., 2006).

1.6.5 Paracetamol

In spite of its widespread use and good effect on pain and fever, but not inflammation, the mechanism of paracetamol is still not known.

1.6.6 Pregabalin

Although introduced as an anti-epileptic drug, the group of drugs that pregabalin belongs to also have an effect on pain. The mechanism, believed to be reduction of neurotransmitter release through binding to the $\alpha 2\delta 1$ subunit of the voltage-gated calcium channels, leads to less neural activity (for review see Sills GJ 2006).

In summary, one aim of this project was to test the predictive value of the rat and mouse models of joint inflammation and surgical OA and the described behavioural endpoints using the NGF/Trk mechanism as a positive target, TRPV1 as a target without effect and compare those to effects by medications used in treatment of arthritis today, e.g. NSAIDs, COX-2 selective antagonists, paracetamol and opioids. In addition, we wanted to explore the role of NGF in arthritis-induced pain and arthritis pathology.

1.7 MUTATIONS OF NGF

Mutations of NGF or the NGF receptor TrkA leads to congenital insensitivity to pain (Nagasako et al., 2003). The resulting condition is named hereditary sensory and autonomic neuropathy (HSAN), and one of these mutations was detected in Sweden; the type V (HSAN V; Minde 2006). Peripheral nerves in homozygous patients show reduced amounts of A δ and C fibres. These patients develop severe joint problems, e.g. Charcot joints, but unlike other types of HSAN, no mental retardation or lack of sweating ability has been found. The exact mutation was found to be a replacement of an amino-acid in the NGF β gene, such that a basic arginine was exchanged for a non-polar tryptophan (Einarsdottir et al 2004), which then produced the NGFR100W protein. Attempts have been made to breed mice with the exact same mutation, but these were not viable unless the mother received normal NGF from gestation until end of lactation, and the pups were given NGF from birth. Even with this treatment, the body weight of these mice pups did not increase after the age of three weeks (Testa et al., 2019). AstraZeneca bred a mouse carrying a similar mutation, the R100E, where arginine was replaced by glutamic acid. These mice, who survive until adulthood, were donated to our group at KI and we were fortunate to be able to test them extensively to evaluate whether these mice show a phenotype similar to the homozygous patients with HSAN V found in the north of Sweden.

2 AIM OF THESIS

The overall aim of the study was to investigate whether behavioural endpoints exist that mimic the movement-induced ongoing pain state in arthritis patients, to give a better predictive value for clinical trials. Two specific aims of this thesis were:

1. Examine if alterations in gait and weight bearing can be used as outcome measure of spontaneous pain in animal models of joint inflammation
 - 1.1. Investigate if and how joint inflammation alters standing weight bearing (static), weight bearing during voluntary and forced locomotion (dynamic) and gait in animal models of arthritis
 - 1.2. Examine if arthritis-induced changes in static and dynamic locomotion and gait are normalized by conventional pain relieving drugs
2. Explore the role of NGF and the ion channel TRPV1 in arthritis-induced spontaneous pain
 - 2.1. Investigate if NGF blockade and TrkA and TRPV1 antagonists reverse arthritis-induced changes in weight bearing and gait
 - 2.2. Investigate if mice with non-functional NGF develop arthritis pathology and/or if they are protected from arthritis-induced changes in gait and weight bearing

3 MATERIAL AND METHODS

3.1 ANIMAL MODELS

3.1.1 Animals

All animal experiments were performed according to regulations and approved by the Swedish local ethical committees. Animals were housed three to six per cage in transparent Macrolon® cages with food and tap water ad libitum and lighting controlled.

Rat strains included Sprague-Dawley (**Paper I, II, III and IV**), Lewis (**Paper III, IV and VI**) and Wistar (**Paper V**), and all studies with rats were done in males. The behavioural experiments were a continuous process that took place over several years. Due to parvovirus infection in the breeding facility at the animal vendor affecting the availability of rat strains, the studies were undertaken in three different rat strains, and Sprague–Dawley rats from two different breeding facilities. Although not examined in a systematic fashion, some variance in duration of ankle joint inflammation in response to the FCA was noted; however the overall behavioural response was similar.

Wild-type C57BL/6JRj mice (**Paper VII and VIII**) and C57BL/6 mice, of both genders, genetically modified by a knock-out of the mNGF, followed by either a knock-in with a wild-type human NGF or with the hNGF containing the mutation (hR100E) in the coding region of the NGF β gene, replacing arginine with glutamic acid (**Paper VIII**) were used.

3.1.2 Chemically-induced monoarthritis models

All joint injections were done while the animals were deeply anaesthetized using 4-5% isoflurane. The injection was completed in less than one minute, and the animals woke up within two to three minutes.

3.1.2.1 Carrageenan-induced monoarthritis

Lambda-carrageenan (Sigma-Aldrich, Sigma Chemicals Co. St Louis, MO, USA) was dissolved in physiological saline. Forty to fifty μ l was injected into one hind limb ankle joint in rats, containing 0.47, 0.94, 1.9, 3.8, 7.5, 15 or 30 mg/ml, for a concentration effect study in **Paper II**, and 7.5 mg/ml was chosen for comparison between ankle and knee joint injection and for pharmacological studies in **Paper I and II**.

3.1.2.2 Complete Freund's adjuvant-induced monoarthritis

FCA/CFA was prepared by adding 100 mg of Mycobacterium tuberculosis (Difco Laboratories) to 12.5 ml of Freund's incomplete adjuvant and dilute to make the different concentrations (0.12, 0.25, 0.50, 1.0, 2.0, 4.0, 8.0 mg/ml) injected with 50 μ l into one hind limb ankle joint in the concentration effect study in **paper II**. CFA (Sigma-Aldrich, Sigma Chemicals Co. St Louis, MO, USA) containing 1.0 mg/ml Mycobacterium tuberculosis was chosen for comparison between ankle and knee joint injection (**Paper II**), and for pharmacological studies in rats (**Paper II, III, IV and V**). In mice, CFA (1.0 mg/ml; Sigma-Aldrich or 10 mg/ml; Chondrex) was injected in volumes of 5 μ l or 10 μ l into one hind limb

ankle joint (**Paper VI**). In **Paper VIII**, CFA (10 mg/ml, Chondrex) was injected into one ankle joint in a volume of 5 μ l, and 10 μ l was injected into one knee joint.

3.1.2.3 Sodium monoiodoacetate-induced monoarthritis

Rats were injected into one hind limb ankle or knee joint with 25 μ l (**Paper III**) or 50 μ l (**Paper VI**) solution containing 2 mg sodium monoiodoacetate (MIA; Sigma) dissolved in physiological saline.

3.1.3 Anterior cruciate ligament transection model

Surgical destabilization of the left knee joint in mice (**Paper VII**) was established by transection of the anterior cruciate ligament (ACLT) under deep anaesthesia, and assured by controlling rotational capacity. Buprenorphine was administered post-surgery as analgesic.

3.2 DRUGS AND DRUG DELIVERY

Table 1. An overview of the drugs used

DRUG	PAPER	SPECIES	ROUTE	DOSES (MG/KG)	DOSES (μ MOL/KG)	INDUCTION AGENT
MEDI-578	Paper IV	Rat	i.v.	0.03, 0.1, 0.3, 3.0, 30 mg/kg		CFA
	Paper VII	Mouse	s.c.	0.3, 1.0, 3.0 mg/kg		CFA
AZ6623	Paper IV	Rat	p.o.		10, 30, 100 μ mol/kg	CFA
					30, 60, 120 μ mol/kg	Carrageenan
AZ7092	Paper IV	Rat	p.o.		1.0, 3.0, 10 μ mol/kg	CFA
					2.0, 6.0, 20, 60 μ mol/kg	Carrageenan
AZD1386	Paper IV	Rat	p.o.		100 μ mol/kg	CFA
NAPROXEN	Paper II	Rat	p.o.		1.0, 10, 100 μ mol/kg	Carrageenan
	Paper IV	Rat	p.o.		7.5, 30, 90 μ mol/kg	CFA
	Paper V	Rat	p.o.	2.5, 7.6, 11.7 mg/kg		CFA
IBUPROFEN	Paper II	Rat	p.o.		30, 100, 300 μ mol/kg	Carrageenan
					100, 300 μ mol/kg	CFA
DICLOFENAC	Paper IV	Rat	p.o.		1.0, 3.0, 10 μ mol/kg	Carrageenan
	Paper II	Rat	p.o.			
ROFECOXIB	Paper I	Rat	p.o.	2.4, 9.4 mg/kg	7.5, 30 μ mol/kg	Carrageenan
	Paper IV	Rat	p.o.		30, 100, 300 μ mol/kg	CFA
VALDECOXIB	Paper IV	Rat	p.o.		10, 30, 100 μ mol/kg	CFA
MORPHINE	Paper I	Rat	s.c.	1.2, 4.8 mg/kg	3.75, 15 μ mol/kg	Carrageenan
OXYCODONE	Paper II	Rat	s.c.		1.0, 3.0, 10 μ mol/kg	Carrageenan
PARACETAMOL	Paper II	Rat	p.o.		1000, 2000, 4000 μ mol/kg	Carrageenan
PREGABALIN	Paper V	Rat	p.o.	10, 30 mg/kg		CFA

3.2.1 Drugs interfering with the NGF-Trk pathway

The monoclonal NGF antibody MEDI-578 was provided by AstraZeneca, MedImmune, dissolved in 50mM sodium acetate and 100 mM sodium chloride and given to rats in a volume of 1 ml/kg intravenously at 0.03, 0.1, 0.3, 3.0 and 30 mg/kg, (**Paper IV**). In studies with mice, MEDI-578 (0.3, 1.0 and 3.0 mg/kg) was injected subcutaneously in a volume of 5 ml/kg (**Paper VII**).

The two pan-Trk antagonists AZ6623 (10, 30, 60, 100 and 120 μ mol/kg) and AZ7092 (1.0, 2.0, 3.0, 6.0, 10, 20 and 60 μ mol/kg) were provided by AstraZeneca, dissolved in 0.5% hydroxypropylmethylcellulose (HPMC) and 0.1% Tween 80, and administered to rats per os in a volume of 5 ml/kg (**Paper IV**).

3.2.2 Drugs interfering with the ion channel TRPV1

AZD1386, a TRPV1 antagonist, was provided by AstraZeneca, suspended in 5% hydroxypropyl- β -cyclodextrin (HBC), 0.2% (w/v) sodiumlaurylsulphate (SDS), 1% (w/v) polyvinylpyrrolidone (PVP), 0.25 sodium citrate in water, and administered to rats per os in a volume of 5 ml/kg at 100 μ mol/kg (**Paper IV**).

3.2.3 Non-steroidal anti-inflammatory drugs

Naproxen (Sigma-Aldrich) was dissolved in physiological saline and administered to rats per os in a volume of 2 ml/kg in doses of 1.0, 10 and 100 μ mol/kg, (**Paper II**), in a volume of 5 ml/kg in doses of 7.5, 30 and 90 μ mol/kg (**Paper IV**), and in a volume of 3 ml/kg in doses of 2.5, 7.6 and 22.7 mg/kg (**Paper V**).

Ibuprofen (synthesized at AstraZeneca) was dissolved in physiological saline and administered to rats per os in a volume of 5 ml/kg in doses of 30, 100 and 300 μ mol/kg (**Paper II**), and ibuprofen (Sigma-Aldrich) was dissolved in physiological saline and administered in doses of 100 and 300 μ mol/kg (**Paper IV**).

Diclofenac (Sigma-Aldrich) was dissolved in physiological saline and administered to rats per os in a volume of 5 ml/kg in doses of 1.0, 10 and 100 μ mol/kg, (**Paper II**).

Rofecoxib (VIOXX, Merck Sharp & Dohme), 2.4 and 9.4 mg/kg (7.5 and 30 μ mol/kg) in suspension and diluted with water, given to rats per os in a volume of 8 ml/kg (**Paper I**). In **Paper IV** rofecoxib (30, 100 and 300 μ mol/kg) synthesized at AstraZeneca was suspended in 1% microcrystalline cellulose/sodiumcarboxymethyl cellulose (MCC/NaCMC)+0.6% soy lecithin (Lipoid S100) in water and given to rats per os in a volume of 5 ml/kg.

Valdecoxib (Kemprotec), 10, 30 and 100 μ mol/kg was suspended in 1% MCC/NaCMC+0.6% soy lecithin (Lipoid S100) in water and given to rats per os in a volume of 5 ml/kg (**Paper IV**).

3.2.4 Opioids

Morphine (Apoteksbolaget, Göteborg, Sweden), which binds preferentially to the μ opioid receptor, in doses of 1.2 and 4.8 mg/kg (3.75 and 15 μ mol/kg), was dissolved in physiological saline, injected s.c. in a volume of 1.0 ml/kg (**Paper I**).

Oxycodone (Sigma-Aldrich), was dissolved in physiological saline and administered to rats subcutaneously in a volume of 2 ml/kg in doses of 1.0, 3.0 and 10 μ mol/kg (**Paper II**).

3.2.5 Other drugs

Paracetamol (Fluka), was dissolved in 0.5 % methyl cellulose and given to rats per os in a volume of 5 ml/kg in doses of 1000, 2000 and 4000 μ mol/kg (**Paper II**).

Pregabalin (Toronto Research Chemicals Inc.), which binds to the $\alpha 2\beta$ subunit of voltage-gated calcium channels to reduce the calcium current after activation (Bauer et al., 2010), was dissolved in physiological saline to get 10 and 30 mg/kg and given per os in a volume of 3 ml/kg in **Paper V**.

3.3 ASSESSMENT OF PAIN-LIKE BEHAVIOUR

3.3.1 Gait and weight bearing during locomotion

Assessment of gait and weight bearing parameters were based on letting animals voluntarily cross an enclosed walkway with a glass floor where light entered the long edge. The projected light is almost entirely reflected within the glass except when an object, such as a paw, touches it. The light is then scattered and produces an illuminated image of e.g. the paw. The light intensity depends on the force of the paw-floor contact and the accumulated light intensity for all pixel in a paw print gives an indirect measure of the weight bearing. A video-camera mounted underneath the glass floor to film the animal as it crossed the walkway gives a number of two-dimensional as well as temporal parameters. In our studies, rats or mice were placed in a goal-cage containing all their cage-mates at the exit of the walkway, and was brought one at a time to the entrance of the walkway. They were allowed to habituate to the walkway and without interference make their own way to the exit in order to find the goal cage and their cage-mates. At least two training sessions were performed before the study was started.

Looking at the films in a pilot study, manual scoring of the weight bearing during locomotion in rats was performed using a visual rating scale, based on the prints and the walking pattern and adapted from the rating scale described by Coderre and Wall (1987) (**Paper II**). In the following studies, two different methods for automated analysis of the results were used; the CatWalk and the PawPrint.

The CatWalk, developed by Frank Hamers and later purchased by Noldus (Noldus Information Technology, The Netherlands), consisted in its first version (**Paper I**) of an analysing tool where the experimenter interactively categorized the points of contact: right/left, fore/hind paw, nose, abdomen, tail and artefacts. This process was later automated, but retained the possibility to edit the resulting classifications (**Paper V, VII and VIII**). Parameters used in our CatWalk studies include: print area, weight load (mean light intensity), stance phase duration, regularity index, walking speed, duty cycle, and weight bearing (Table 2). The PawPrint (developed by AstraZeneca, Sweden) was a custom made computer program for rats only (**Paper II, IV and VI**), with an automated analysis of the crossing. The program immediately calculated results and displayed a picture of the prints in false colours (one colour for each paw) which allowed the experimenter to make a quality check of the results. Editing of the classifications were not possible. If results were not acceptable, the rat was allowed to walk again across the walkway to give at least three paw placements by each non-arthritic paw. Parameters used in our PawPrint studies include weight bearing and regularity index.

Table 2. Description of parameters from the CatWalk

PARAMETER	PAPER	DESCRIPTION	CALCULATION
WEIGHT BEARING	Paper II, IV, V, VII, VIII	Calculated force of a paw against the glass floor	Max contact area x Mean intensity
WEIGHT BEARING (%)	Paper II, IV, V, VI, VII, VIII	Weight bearing of a paw relative to all paws	$(\text{Calculated weight bearing}_{\text{paw}} / \Sigma \text{calculated weight bearing}_{\text{all paws}}) \times 100$
GUARDING INDEX (%)	Paper II, IV, V, VII	Difference in percent weight bearing between the non-injured hind paw and the injured hind paw	$\text{Weight bearing } (\%)_{\text{non-injured paw}} - \text{weight bearing } (\%)_{\text{injured paw}}$
PRINT AREA	Paper I	The entire surface area of the complete print	
LIGHT INTENSITY	Paper I	The average max light intensity (arbitrary unit 0-255) of all pixel in a print area, which is an indirect measure of weight load put on the glass floor	
DURATION OF STANCE PHASE	Paper I, VII	Duration of contact of a paw with the glass floor	
DUTY CYCLE	Paper V, VII, VIII	The duration of the stance phase as a percentage of the entire step cycle (stance + swing)	$[\text{Stance time} / (\text{Stance time} + \text{Swing time})] \times 100$
STRIDE LENGTH	Paper VIII	The distance between successive placements of the same paw	
SWING SPEED	Paper VIII	The speed of the paw as it is moved from one placement to the next	
WALKING SPEED	Paper V, VII	Average speed of the selected steps in the walkway crossing	
REGULARITY INDEX (%)	Paper I, II, V, VII, VIII	The number of normal step sequence patterns (NSSP) relative to the total number of paw placements (PP)	$[(\text{NSSP} \times 4) / \text{PP}] \times 100$

3.3.2 Stationary weight bearing

Scoring of the stationary weight bearing in rats in an acrylic test box filmed from underneath was performed using an extended version of the visual rating scale, previously described by Coderre and Wall (1987) (**Paper II**).

Mice were tested for stationary weight bearing using the Advanced Dynamic Weight Bearing (DWB; Bioseb, France). This device consists of an 11cm x 11cm floor, with transparent walls and a removable top equipped with a camera. The floor is covered by a pressure mapping sensor mat (TecScan) containing 1,936 SenselsTM which indirectly register the weight of each paw. The mouse was allowed to move freely within the enclosure for five minutes, but measurements were only obtained when it was immobile, and the total time giving results ranged between 1.5 and 3 minutes. Weight bearing in grams was given for each paw, and the relative weight bearing in percent of the total body weight calculated. In addition, the duration of floor contact in percent of the entire measurement time is given (**paper VII and VIII**).

3.3.3 Static weight bearing

Using the Incapacitance tester (Linton Instrumentation, UK) the static weight bearing of the two hind paws was assessed in rats. The animals were placed in restrainers with the front paws on a ramp and their hind paws resting on two separate scales which measured the load of each paw. The average of five consecutive recordings, each lasting three seconds, were calculated, and weight bearing is shown as percent of both hind paws (**Paper V and VI**).

3.3.4 Mechanical sensitivity

Optihair von Frey filaments (MARSTOCK nervtest, Germany) with logarithmically incremental stiffness were used to assess mechanical sensitivity in both rats and mice (**Paper V and VIII**). The animals were placed in individual enclosures on a wire mesh floor. Rats were habituated to the observation boxes for 10 minutes before testing, while mice needed habituation for at least 30-40 minutes. Filaments were applied perpendicular to the plantar surface from underneath the grid floor until slight buckling of the filament. The 50% withdrawal threshold was calculated using the up/down method (Chaplan et al., 1994, as adapted from Dixon 1980); in the event of a paw withdrawal a positive response was noted, and a filament with a lower bending force was subsequently applied; in the case of no paw withdrawal a filament with a higher bending force was applied.

3.3.5 Cold sensitivity

Acetone was applied from underneath to the plantar surface of the hind paw to assess cold sensitivity in mice (**Paper VIII**). After habituation in enclosures on a wire mesh floor, approximately 10 µl of acetone was applied and the time spent reacting (e.g. licking, biting or shaking) was measured over 60 seconds. This procedure was repeated three times and the mean value was calculated.

3.3.6 Thermal sensitivity

Using the Hargreaves' apparatus, mice were tested for latency to withdrawal from a beam of radiant heat stimulus directed from underneath onto the plantar surface of the hind paw (**Paper VIII**). Before testing, the mice were habituated in Plexiglass enclosures on a glass surface. This procedure was repeated three times for each hind paw, and the mean value was calculated.

3.3.7 The formalin test

The formalin test in mice consists of recording the behaviour subsequent to dorsal subcutaneous injection into one hind paw of 10 µl formalin 5% in physiological saline (**Paper VIII**). The injection was performed under isoflurane anaesthesia, and the animals were directly afterwards put into Perspex enclosures (13 cm x 13 cm) resting on a pane of Lexan (polycarbonate), and filmed from the front with a mirror fitted at an angle below the box to make it possible to observe in detail what the animal was doing also from underneath. The amount and time spent flinching or licking/biting the injected paw was recorded in 12 blocks of 5 minutes for a total of one hour.

3.4 COLLECTION OF FLUIDS AND TISSUES

3.4.1 Blood

3.4.1.1 Blood collected for exposure analysis in pharmacological effect studies

Blood was collected via heart puncture into test tubes containing EDTA at termination of testing from anaesthetized rats included in the behavioural effect studies and centrifuged to get plasma in **Paper II and IV**. To get information of the plasma exposure also at the time-points of behavioural testing, blood was obtained from the tail vein from three satellite animals per treatment group. Samples were kept on ice until centrifugation at 4°C and supernatants from all samples were stored at -80°C until analysis. Analysis of drug exposure was performed using a bioanalytical method that was developed for each drug to determine concentrations in the low nanomolar range in plasma samples by reversed-phase liquid chromatography and electrospray tandem mass spectrometry.

3.4.1.2 Blood collected for analysis of inflammatory biomarkers

Blood was collected via heart puncture and divided into two tubes; one without additives to yield serum, and another containing EDTA to yield plasma (**Paper III**). To get serum, the blood was kept in room temperature until clotting (approximately 30 minutes) and then centrifuged at 4°C and supernatants from all samples were stored at -80°C until analysis.

3.4.2 Synovial fluid

Synovial fluid was collected immediately following sacrifice of the rat by intra-cardiac injection of pentobarbital (**Paper III, IV and VI**). The skin was cut transversally above the ankle or the knee joint and the ligament above the joint punctured by a scalpel. The synovial fluid was collected by rinsing the joint cavity four subsequent times by a pipette containing 25 µl 0.05M EDTA (Sigma, pH 7.5). Samples were kept on ice until centrifugation at 4°C and supernatants from all samples were stored at -80°C until biomarker analysis.

3.4.3 Hind leg joints

Ankle or knee joints were taken from rats in **Paper VI**, and from mice in **Paper VII**. Joints were taken immediately after euthanasia, skin and soft tissues carefully removed and the diaphyses carefully opened proximal and distal of the joint to ensure rapid internal fixation. The joints were then fixated in 4% phosphate buffered paraformaldehyde (Histolab Products AB) for at least 24 hours. Decalcification of the mineralized tissue was done in EDTA (0.1 mol/l, pH 7.4; Sigma-Aldrich) for at least two weeks before dehydration and embedding in paraffin.

3.4.4 Nervous tissue and skin

The plantar skin from hind paws, the sciatic nerves and the L5 dorsal root ganglion were harvested in **Paper VIII**, from mice deeply anesthetized with isoflurane and transfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. The tissue was post-fixed and cryoprotected in 20% sucrose in PBS until embedding in paraffin.

3.5 BIOCHEMICAL BIOMARKER ANALYSIS

3.5.1 Colometric assay

In **Paper III and VI**, L(+)-Lactate levels were assessed in synovial fluid from rats. Samples were diluted up to 1:32 with the provided kit buffer before transferral to a 96-well microplate. After mixing with an equal volume of the reaction mix provided by the vendor, the samples were incubated for 30 minutes at room temperature before the microplate was read at 570 nm on a Spectramax 340 (Molecular Devices). By extending the standard curve and converting the concentrations to millimoles per litre, the colorimetric assay (Biovision) was slightly modified, and the lower limit of quantification (LOQ) was 0.02 mmol/l.

3.5.2 Immunoassay

3.5.2.1 NGF

Levels of rat NGF were assessed in synovial fluid in **Paper IV**. This immunoassay was developed using electro-chemiluminescence (ECL) technique, where plates were coated with rat beta-NGF antibody, then blocked with 3% bovine serum albumin for 30 minutes before washing. Synovial fluid samples were diluted 1:4 in assay diluents and added to the plates to incubate for 2 hours. Recombinant rat beta-NGF was used as a calibrator. After washing, rat Beta-NGF biotinylated antibody provided with MSD Sulfo TAG (Meso Scale Discovery) was dispensed to all wells and the plates were incubated to an additional 1.5 hours. Once again the plates were washed, followed by adding read buffer to the wells and subsequent analysis on the SECTOR Imager (SI6000, Meso Scale Discovery). The LOQ was 9.77 pg/ml.

3.5.2.2 Cytokines and chemokines

Levels of MCP-1, MIP-3 α , KC/GRO and IL-6 were assessed in synovial fluid, serum and EDTA plasma from rats in **Paper III**, and in synovial fluid in **Paper VI**. Plasma and serum samples were diluted 1:2, and synovial fluid was diluted 1:4 in assay diluents and added to the plates. Custom-made immunoassay kits (Meso Scale Discovery), tested and validated for all matrices following the manufacturer's instructions were used. Concentrations of IL-1 β and TNF, assessed in synovial fluid, serum and EDTA plasma from rats in **Paper III**, were assessed using commercially available multiplex immunoassay kits. The LOQ, corrected for dilutions, were 624 pg/ml for MCP-1, 39 pg/ml for MIP-3 α , KC/GRO, IL-1 β and TNF, and 79 pg/ml for IL-6.

3.6 IMMUNOHISTOCHEMISTRY

Joints imbedded in paraffin were cut in 4-6 μ m serial sections, mounted on glass slides and stained with Safranin-O (**Paper VI**) to detect loss of proteoglycan in the rat cartilage extracellular matrix, done under light microscopic analysis using a Zeiss Axioplan 2 microscope, or Sag-O-Fast (**Paper VII**) followed by a semi-quantitative scoring according to Osteoarthritis Research Society International (OARSI) histological scoring for mice.

Nervous tissue cut in 12 μm serial sections were mounted on glass slides and rehydrated with PBS, permeabilized with 0.2 Triton X in PBS, and incubated with 5% normal goat or donkey serum in 0.2% Triton X/PBS to block non-specific binding (**Paper VIII**). The sections were subsequently incubated overnight at 4° with different primary antibodies:

Primary antibody	Vendor	Catalog number	Dilution
Rat anti-TrkA	R&D Systems	AF1056	1:100
Rabbit anti-PGP 9.5	Abcam	Ab37188	1:1000
Rabbit ant-CGRP	Sigma	C8198	1:2000
Mouse anti-Tuj-1	Promega	G7121	1:2000

To visualize the immunoreactivity, the secondary antibodies were conjugated to Alexa 488 (1:300; Life Technologies), Alexa 555 (1:300; Life Technologies), Cy2 (1:300; Jackson Laboratory) or Cy3 (1:600; Jackson Laboratory). An inverted fluorescence microscope was used to acquire the images.

The number of fibres/ μm^3 and the fibre length/ μm^3 was assessed in glabrous skin using the Fibre Nerve Counting Rules (33, 34) and quantification of sciatic nerve fibres labelled with the antibodies described above was carried out using a custom script; 20 lines at even intervals throughout the region of interest were drawn perpendicular to the direction of axons and the pixel intensities along each line were plotted. The intensity matrices were then processed through a peak-detection algorithm to detect the labelled nerve fibres represented as positive intensity peaks on individual line plottings. The proportion of CGRP positive fibres in Tuj1 positive nerve fibres was analysed in four to five sections per animal (with a minimum of 30 μm apart) and the average value for each animal was calculated. For quantifying the proportion of TrkA positive neurons in L5 DRG, the number of TrkA positive neurons was counted and divided by the number of PGP9.5 (pan-neuronal marker) positive neurons. Four to five sections per animal (with a minimum of 30 μm apart) were analysed and the average value for each animal was calculated.

3.7 ASSESSMENT OF INFLAMMATION

The diameters of ankle or knee respectively were assessed in **Paper III, VI, VII and VIII**, taking the mean of 3 measurements from a three-button digital calliper (Limit, Alingsås, Sweden).

3.8 STATISTICAL ANALYSIS

Two-way analysis of variance (ANOVA) was used for behavioural studies where changes over time were compared. Duncan's test was used as post-hoc test in **Paper I**, and Bonferroni's multiple comparison test in **Paper II, IV, VI, VII and VIII**. In **Paper V**, the non-parametric Mann-Whitney test for each time point was used instead, as not all time points included all rats and the variances were not equally distributed.

When comparison was made between three or more groups, one-way ANOVA followed by Dunnett's multiple comparison was used in **Paper II and VII**. When analysing the effects of treatment in the carrageenan-induced monoarthritis in **Paper IV**, one-way ANOVA was followed by Bonferroni's t-test versus the vehicle group, and levels of NGF in synovial fluid were analysed by the non-parametric Kruskal-Wallis one-way ANOVA followed by Student-Newman-Keuls method. In **Paper V** the Kruskal-Wallis test was used followed by Dunn's multiple comparison test. Levels of L(+)-Lactate in **Paper VI** were analysed using Kruskal-Wallis test with Mann-Whitney's post hoc test.

Differences between two groups were analysed by student's t-test (**Paper VII and VIII**). However, data in **Paper III** from the rat plasma and serum groups were log-transformed before comparison with the t-test, whereas comparison between cytokine levels in human patients and healthy controls was done using the Mann-Whitney *U*-test.

In **Paper II**, the non-parametric Kendall rank correlation coefficient test was used to compare visual scoring of static versus dynamic paw pressure in rats. Pearson's correlation coefficient with the corresponding *P*-value was used in **Paper III and VII**.

Data in **Paper I, II and VIII** are presented as the mean \pm standard error of the mean (SEM). In **Paper IV**, the behavioural data are presented as the mean \pm SEM, and the levels of NGF in synovial fluid are presented as the mean \pm 95% confidence interval (CI). Data in **Paper III, V, VI and VII** are presented as the mean \pm 95% CI. Results from analysis of biochemical mediator levels in **Paper VI** are presented as individual levels and medians. Statistical significance is shown as **P* < .05, ***P* < .01, ****P* < .001. Software for statistical tests were SigmaPlot 11.0 (**Paper I, II and IV**), GraphPad Prism 6.00, 6.03 or 7 (**Paper III, V, VI, VII and VIII**), and SAS/STAT 9.3 (**Paper III**).

4 RESULTS

4.1 GAIT AND WEIGHT BEARING AS OUTCOME MEASURE OF SPONTANEOUS PAIN IN ANIMAL MODELS OF ARTHRITIS

4.1.1 Carrageenan, CFA- and MIA-induced monoarthritis in rats

Paper I, II, IV, V, VI

The effects of a short-lasting inflammatory model – carrageenan-induced monoarthritis – was tested to establish its suitability for further studies. The effects on weight bearing while standing (static) was tested using the Incapacitance tester, where naïve rats put close to 50% of the weight on each hind paw. Rats with carrageenan intra-articular ankle joint injection (7.5 mg/ml; 375 µg in 50 µl) reduced the weight bearing of the injected paw to values between 31% and 33%, assessed at 3, 5 and 24 hours after injection (**Paper II**, Fig 10).

Using the CatWalk setup, the effects of carrageenan injected into the ankle joint on gait and weight bearing during locomotion was assessed. Compared to saline injected animals, the monoarthritic rats showed significantly reduced print area, mean weight load (light intensity) and duration of stance for the injected paw at all times tested, and loss of inter-limb coordination (**Paper I**, Fig 3).

As the CatWalk was not commercially available at the time, AstraZeneca built a similar equipment in-house, based on the same principles, and named it PawPrint. The analysis algorithms in the PawPrint allowed for immediate automated analysis, with results shown as a picture of the prints in false colours for easy identification (Figure 3), and thus allowed for a quality check of the results (**Paper II**, Fig 1).

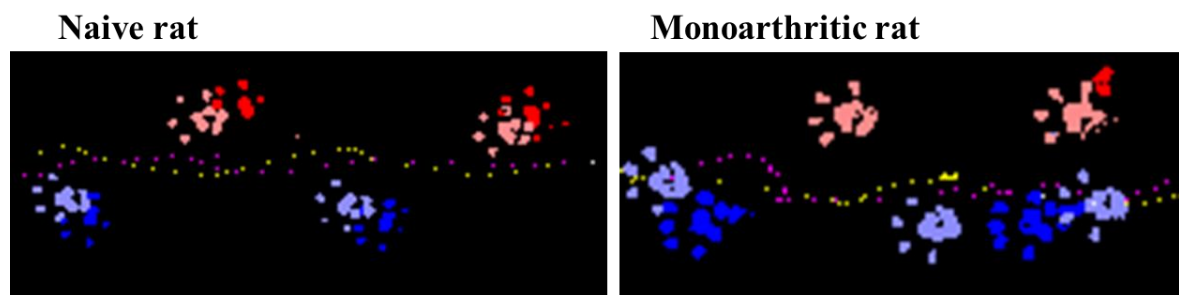


Figure 3. Picture of prints from a naïve rat and a rat with monoarthritis from the PawPrint analysis. Prints from the left side are coloured red, and prints from the right side blue. Light colours show front paws, while dark colours show hind paws.

The parameters included print area and light intensity from all prints. Using accumulated light intensity from the number of pixel in a print area we calculated weight bearing. The four median values from prints of the four paws were chosen from a walkway crossing, and the relative contribution for each paw was calculated as per cent of the sum of weight bearing values for all paws. The difference in percent weight bearing between the two hind paws was calculated to show the shift in weight bearing from the inflamed hind paw to the non-inflamed hind paw, and this new parameter was named guarding index. Using the PawPrint setup a range of seven concentrations of carrageenan injected into one hind paw ankle joint was tested. The results showed that the reduction in weight bearing of the injected hind paw was compensated

by increased weight bearing of the front paw on the same side as the injected paw, and by the contralateral hind paw (**Paper II**, Fig 6). The guarding index, showing values close to zero in naïve or saline-injected rats, increased significantly and concentration-dependently already from the low amount of 0.024 µg at 5 hours after injection, and lasted for up to 48 hours after the high amount of 1500 µg (**Paper II**, Fig 7). Regularity index, close to 100% in naïve or saline-injected rats, demonstrate that those animals use all four paws in a coordinated fashion during locomotion. Carrageenan concentrations above 1.9 mg/ml (0.095 µg) significantly reduced the regularity index, but these effects were normalized at 24 hours after injection.

Subsequently FCA, in concentrations between 0.12 to 8.0 mg/ml (0.006 to 400 µg in 50 µl), was used to induce ankle joint inflammation and effects measured with the PawPrint setup. Similar effects regarding the four paw's weight bearing as after injection of carrageenan were found (**Paper II**, Fig 4), but the increase of guarding index was significant for all concentrations (even for Freund's incomplete adjuvant) and lasted for more than 10 days for the high concentration (**Paper II**, Fig 5). Regularity index was decreased significantly for all concentrations at some time point, but again lasted for a shorter time period than did effects on weight bearing.

Next, MIA in a concentration (2 mg in 50 µl; 2000 µg) that had previously been shown to induce weight bearing deficits (Combe et al., 2004) was injected into the ankle joint and tested for effects on weight bearing during locomotion up to 28 days after injection. Surprisingly, a reduction in weight bearing of the injected paw, compensated by an increase in the contralateral hind paw, was only seen at one day after induction (**Paper VI**, Fig 1A). As published results are all based on the effects of MIA intra-articular injection into the knee joint, this was then tested and showed significant effects on weight bearing during locomotion, but which started only at three weeks after induction. In contrast, static weight bearing was significantly reduced from day 1, not on day 7, but lasted for the rest of the 28 days test period (**Paper VI**, Fig 2B).

To further elucidate the differences between monoarthritis induced in the ankle joint compared to the knee joint, both carrageenan (375 µg), FCA (50 µg) and MIA (2000 µg) groups with knee joint injection were included in the studies using ankle joint injection. The effects of the joint inflammation on weight bearing during locomotion were more pronounced when injecting into the ankle joint for all agents (Figure 4), and the responses after intra-articular injection of carrageenan and FCA were stronger. Only MIA-induced knee monoarthritis in rats developed a second phase (**Paper VI**, Fig 2A), at the time when cartilage breakdown has been reported (Guinkamp et al., 1997; SBU, 2006), and no tendency of a second phase was found after one single joint injection of carrageenan or FCA.

To compare the usage of the injected paw when standing to that when walking, rats with monoarthritis induced by injection of Freund's complete adjuvant were filmed from underneath, both in a box (length 30cm, width 20cm, and height 18cm) and in a walkway (length 1m, width 10cm, open top), and later scored manually for guarding/paw pressure with the rating scale described by Coderre and Wall 1987. Results showed that guarding scores were always higher as the rats walked compared to when they were standing still (**Paper II**, Fig 3).

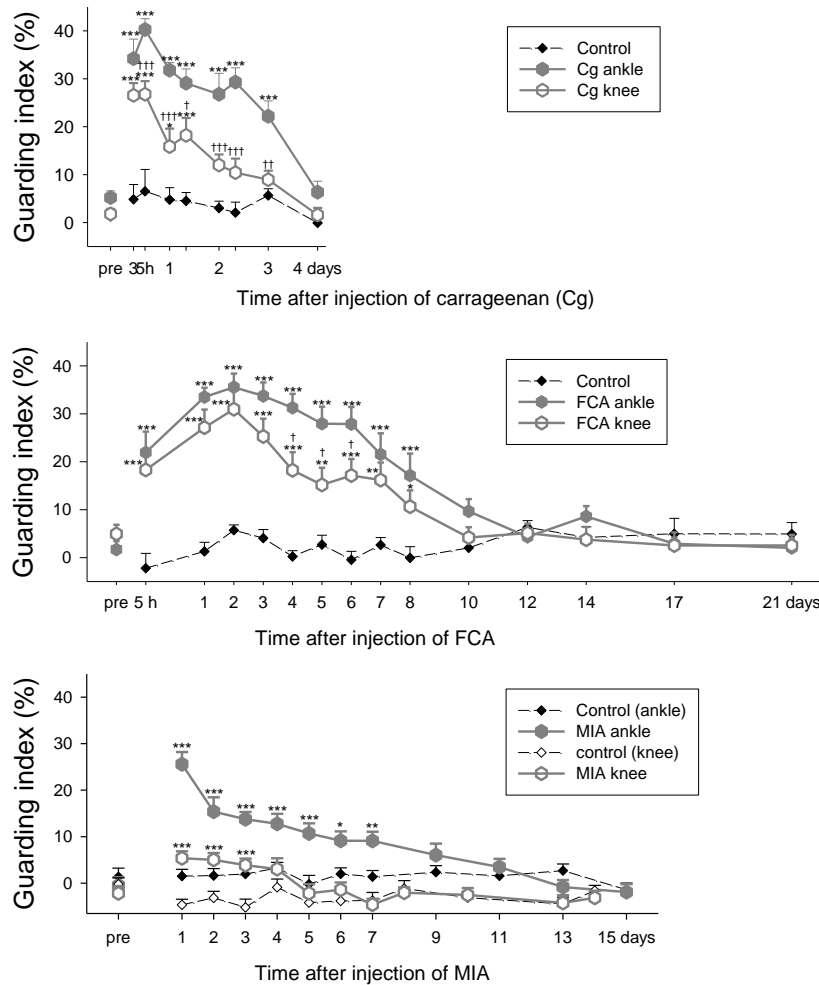


Figure 4. Injection site of carrageenan (7.5 mg/ml, upper panel), FCA (1.0 ml/ml, middle panel) and MIA (2 mg, lower panel) plays a role for magnitude of response on guarding index assessed in the PawPrint. Bonferroni's test subsequent to 2-way ANOVA: * $P < .05$, ** $P < .01$, *** $P < .001$ compared to the control group at the same time point. Data shown as mean \pm SEM, $n = 10$ per group. (Adapted from **Paper II**, Figs 8 and 9)

4.1.2 Pro-inflammatory biomarkers in synovial fluid and blood after CFA- and MIA-induced monoarthritis in rat

Paper III, IV, VI

To more closely study also the levels of inflammatory factors in the joint, and compare to levels in the blood, we performed an additional study. Here we used FCA (50 μ g) and MIA (2000 μ g) to induce monoarthritis, and plasma, serum and synovial fluid was collected from rats at several time-points and analysed for contents of IL-1 β , IL-6, KC/GRO, MCP-1, MIP-3 α , TNF and the indicator of a successful induction of inflammation; L(+)-lactate (Finn and Oerther, 2010). All markers showed elevated levels in synovial fluid from monoarthritic rats compared to control groups, although with considerably lower magnitude in the MIA injected rats, where indications of a biphasic pattern appeared (**Paper III**, Figs 1 and 2). Levels of KC/GRO and MIP-3 α in serum from the FCA model, and IL-6 in serum from the MIA model followed the pattern of levels found in the synovial fluid.

To compare some of the same inflammatory factors in ankle versus knee intra-articular injection of MIA (2000 µg), synovial fluid was collected at termination of the 28 day behavioural MIA study. Results confirmed the disparate injection site profiles; thus knee injection caused elevated levels of MCP-1, MIP-3 α , KC/GRO and IL-6 whereas ankle injection resulted in inflammatory factors at levels similar to those in naïve rats at 28 days after injection (**Paper VI**, Fig 4). Additionally, joint pathology showed similar differences; only injection of MIA into the knee joint caused severe cartilage erosions, exposure of subchondral bone and osteophyte formation, while ankle joint injection caused very mild changes in a few rats (**Paper VI**, Figs 5 and 6).

4.1.3 CFA-induced monoarthritis in mice

Paper VII, VIII

At Karolinska Institutet, the focus for me has shifted to using mice as test species.

Tests with monoarthritic mice assessing stationary weight bearing on the DWB showed that this setup produced data revealing a robust separation between monoarthritic (CFA; 10 and 100 µg) compared to control animals. Naïve mice exerted significantly more weight on hind paws ($\approx 80\%$) than on front paws ($\approx 20\%$), and hind paws stayed in contact with the floor for longer. Mice with CFA injected into the ankle joint of one hind paw showed reduced stationary weight bearing of the injected paw, outlasting the 2-week testing period. A corresponding increase of weight bearing was seen for the front paw on the injected side, and to some degree also for the non-injected hind paw. Similarly, the duration of floor contact was reduced for the injected hind paw and increased for the front paw on the injected side (**Paper VII**, Fig 2).

Using the CatWalk setup to test mice induced with CFA monoarthritis (10 and 100 µg) for changes in gait and weight bearing during locomotion, similar features as those found in rats were found; the weight bearing of the injected paw was drastically reduced, while the other paws compensated for the loss of weight bearing. However mice compensated by putting more weight not only on the contralateral hind paw, but also on the contralateral front paw, in contrast to rats that compensate with the contralateral hind paw and ipsilateral front paw. Moreover, the high amount CFA (100 µg) caused a prolonged behavioural effect, and no return to normal usage of the paws was seen over the two week test period (Figure 5).

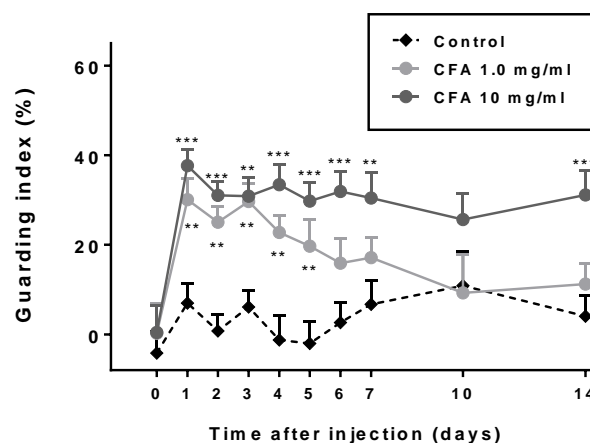


Figure 5. Guarding index assessed on the CatWalk in mice with injection of FCA (10 and 100 μ g) into one hind paw ankle joint. Bonferroni's test subsequent to 2-way ANOVA: * $P < .05$, ** $P < .01$, *** $P < .001$ compared to the control group at the same time point. Data shown as mean \pm SEM, $n = 10$ per group. (Adapted from **Paper VII**, Fig 3)

In **Paper VIII**; manuscript, we tested mice with the human wild-type NGF gene, injected with CFA (10 mg/ml; 5 μ l or 10 μ l into the ankle or knee joint respectively) for effects on stationary (DWB) and dynamic (CatWalk) weight bearing. These mice showed similar response to those tested for Paper VII; significant reduction of the injected hind leg's weight bearing (**Paper VIII**, Figs 6 and 7; manuscript).

4.1.4 Anterior cruciate ligament transection in mice

Paper VII

During the eight weeks of testing mice with sham surgery for stationary weight bearing on the DWB, this control group resembled previous data; $\approx 80\%$ weight bearing was on the hind paws and $\approx 20\%$ was on the front paws. The duration of floor contact showed a similar pattern.

After ACLT surgery, the injured paw exerted less body weight for two weeks and then returned to normal. The weight bearing decrease was compensated by a small increase on the non-injured hind paw and on the front paw on the side of the surgery. However, the small increase in weight bearing of the non-injured hind paw remained evident until the end of the study, and resulted in a significant increase of the stationary guarding index which correlated with the degree of structural changes in the injured joint (Figure 6). The duration of floor contact was affected for one week post-surgery only (**Paper VII**, Fig 1; manuscript).

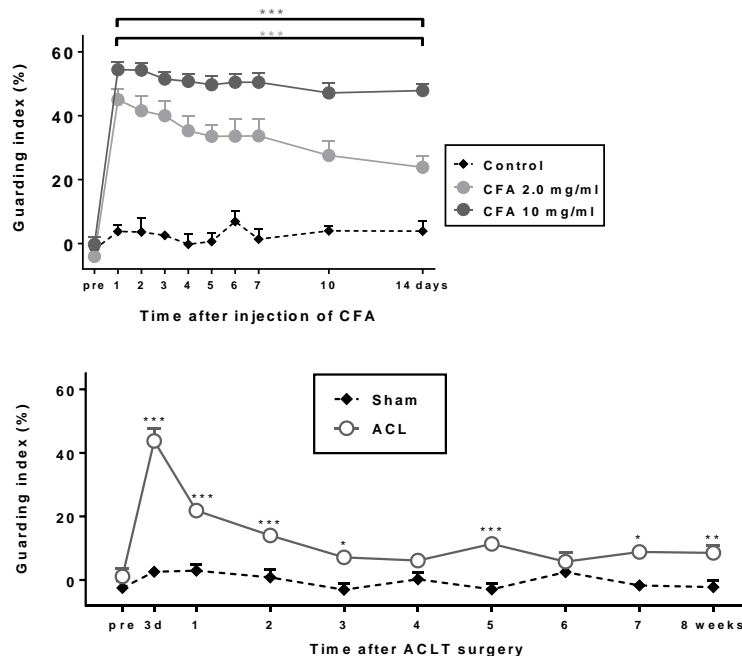


Figure 6. Guarding index assessed on the DWB in mice with injection of FCA (10 and 100 μ g) into one hind paw ankle joint (upper panel), and in mice with ACLT in one hind leg knee joint (lower panel). Bonferroni's test subsequent to 2-way ANOVA: * $P < .05$, ** $P < .01$, *** $P < .001$ compared to the control group at the same time point. Data shown as mean \pm SEM, $n = 10$ per group. (Adapted from **Paper VII**, Figs 1 and 2)

4.2 ARTHRITIS-INDUCED CHANGES IN GAIT AND WEIGHT BEARING ARE NORMALIZED BY CONVENTIONAL PAIN RELIEVING DRUGS

Paper I, II, IV, V

When assessed on the PawPrint, rats with monoarthritis induced by carrageenan or CFA reached guarding index levels of about 30%, from levels around 0% before induction and in control rats; naïve or injected by saline into the ankle joint.

In rats with carrageenan induced monoarthritis the drugs were given before testing at a time-point which produced the highest plasma concentration at testing, as established by the DMPK department at AstraZeneca. Single administration of the tested compounds produced dose-related reductions in the response to carrageenan as measured by the guarding index (Figure 7). Significant effects were seen at doses of 10 (naproxen), 100 (ibuprofen), 1 (diclofenac), 3 (oxycodone) and 2000 (paracetamol) $\mu\text{mol/kg}$, corresponding to 2.3 (naproxen), 23 (ibuprofen), 0.30 (diclofenac), 1.1 (oxycodone) and 300 (paracetamol) mg/kg . All compounds produced dose related total plasma exposures (**Paper II**, Table 2).

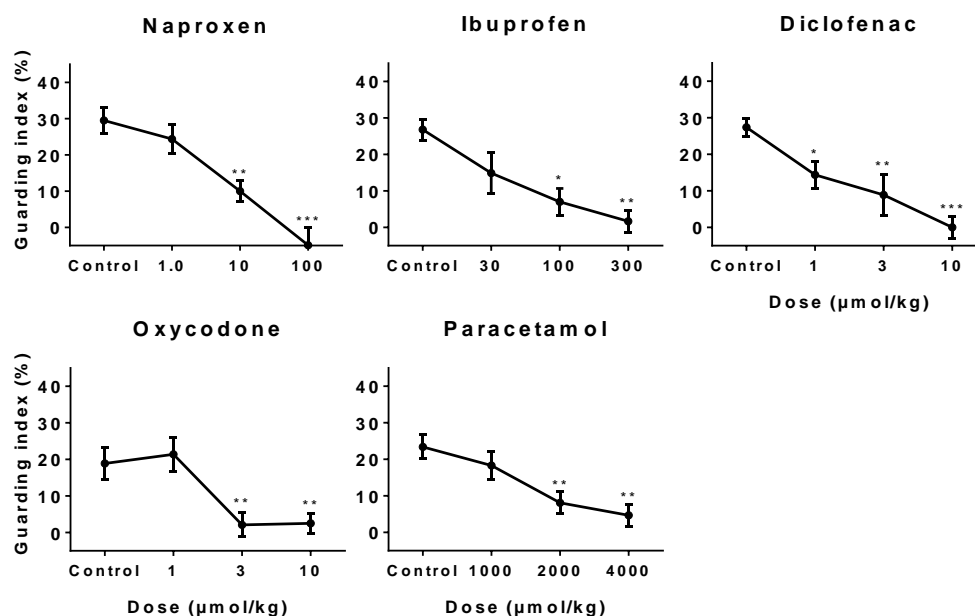


Figure 7. Guarding index assessed on the PawPrint in rats with carrageenan monoarthritis (7.5 mg/ml) of one hind paw ankle joint. Dunnett's test subsequent to 1-way ANOVA: * $P < .05$, ** $P < .01$, *** $P < .001$ compared to the control group. Data shown as mean \pm SEM, $n = 8$ -10 per group. (Adapted from **Paper II**, Table 2)

Administration of the non-selective COX inhibitors naproxen and ibuprofen (**Paper IV**, Fig. 6A–D) also attenuated the increase in guarding index induced by FCA ankle injection. The highest doses of naproxen (90 mmol/kg) and ibuprofen (300 mmol/kg) normalized guarding index on day 3 of treatment compared to the respective vehicle treated groups. The two COX-2-selective inhibitors valdecoxib and rofecoxib (**Paper IV**, Fig. 7A–D) showed similar normalising effects on guarding index (Figure 8). The effects were statistically significant at 3 days after treatment start compared to vehicle treated rats. But the degree of normalization is

not as high as that seen in the carrageenan monoarthritis. The total plasma concentrations remained stable during the test period for all COX inhibitors (**Paper IV**, Table1).

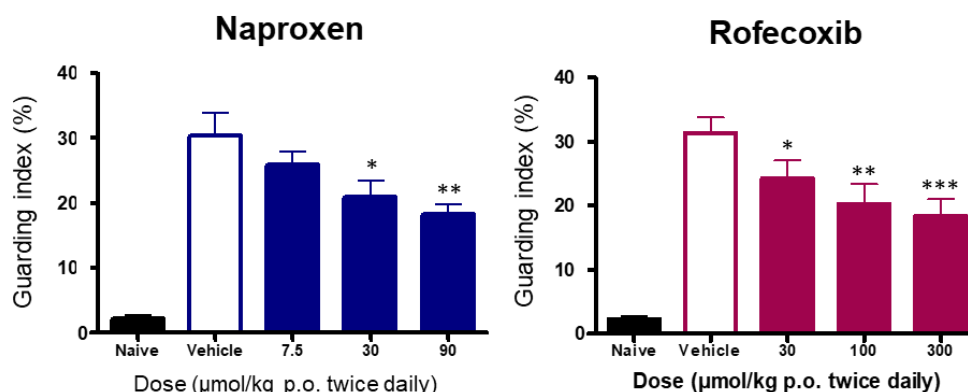


Figure 8. Guarding index after 3 days of daily treatment, assessed on the PawPrint in rats with FCA monoarthritis (50 μg) of one hind paw ankle joint. Bonferroni's test subsequent to 2-way ANOVA was performed on the total test time: * $P < .05$, ** $P < .01$, *** $P < .001$ compared to the control group. Data shown as mean \pm SEM, $n = 11-12$ per group. (Adapted from **Paper IV**, Figs 6 and 7)

4.3 NGF BLOCKAGE AND TRKA ANTAGONISTS REVERSE ARTHRITIS-INDUCED CHANGES IN WEIGHT BEARING AND GAIT, BUT A TRPV1 ANTAGONIST FAILED TO DO SO

Paper IV, VII

First we assessed the release of NGF in synovial fluid of the joint subsequent to induction of inflammation with FCA (50 μg), and compared levels to those in the synovial fluid from saline injected rats. NGF levels were significantly elevated in synovial fluid from intra-articular ankle joint FCA injected rats, reaching maximum at days one and two, but were still significantly increased on days five and ten after the joint injection (**Paper IV**, Fig 1).

To investigate whether blockade of NGF or its receptor TrkA could normalize weight bearing during voluntary locomotion after induction of joint inflammation, we tested two pan-Trk inhibitors - AZ6623 and AZ7092 – in rats after ankle joint injection of carrageenan (375 μg), using the PawPrint setup. Both showed normalization of the guarding index in a dose-related manner, but couldn't completely restore the weight bearing to that of naïve rats (Figure 9).

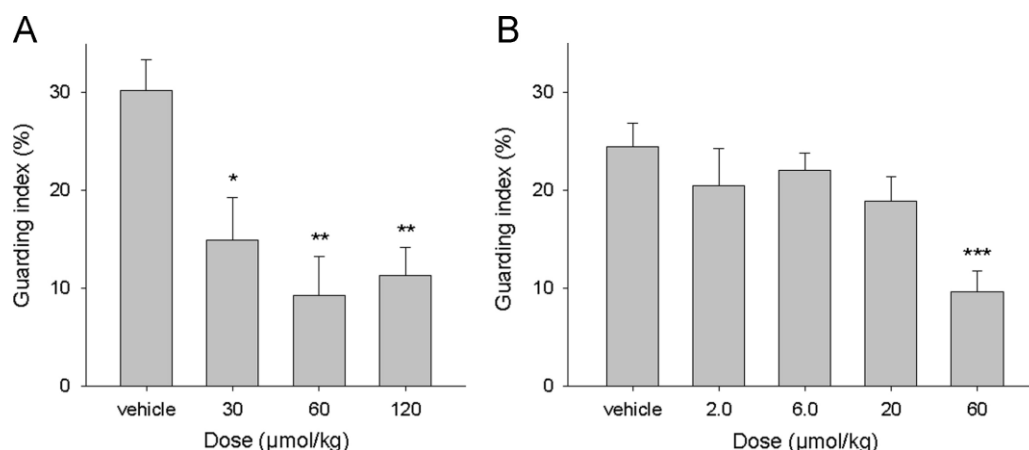


Figure 9. Effects of the pan-Trk antagonists AZ6623 and AZ7062 on carrageenan-induced changes in guarding index are shown in (A) and (B) respectively. AZ6623 ($n = 8/\text{group}$) and AZ7092 ($n = 10\text{--}12/\text{group}$) were given as a single administration orally, 1 h after ankle joint injection of carrageenan, and testing was performed 2 h later. Data shown as mean \pm SEM, and analysed by 1-way ANOVA followed by Bonferroni's t-test. $*P < .05$, $**P < .01$, $***P < .001$ compared to the respective carrageenan injected vehicle group. (From **Paper IV**, Fig 4)

Next the pan-Trk inhibitors AZ6623 and AZ7092, and the anti-NGF antibody MEDI-578 were tested in the PawPrint setup for effects on FCA-induced guarding index during voluntary locomotion (Figure 10). Here, one of the pan-Trk inhibitors was slightly more efficacious than the NGF antibody, but all were more efficacious than the previously tested NSAIDs. Thus, the normalization of guarding index compared to vehicle treated rats reached approximately 60 to 80% (Figs 2 and 3 in paper IV).

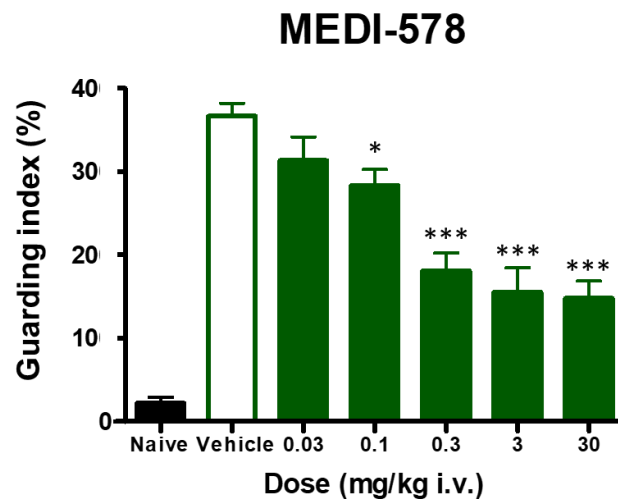


Figure 10. Guarding index at 3 days after one single intravenous injection of MEDI-578, assessed on the PawPrint in rats with FCA monoarthritis (50 μg) of one hind paw ankle joint. Bonferroni's test subsequent to 2-way ANOVA was performed on the total test time: $*P < .05$, $**P < .01$, $***P < .001$ compared to the control group. Data shown as mean \pm SEM, $n = 11\text{--}12$ per group. (Adapted from **Paper IV**, Fig 2)

In contrast, when testing a TRPV1 inhibitor at a high dose, no effect was observed (Figure 11).

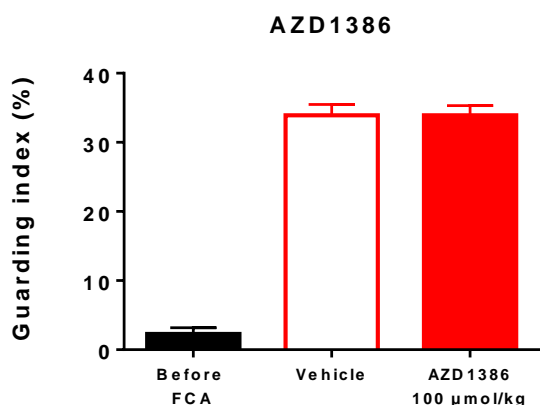


Figure 11. Guarding index before and at 3 days after twice daily oral treatment of AZD1386, assessed on the PawPrint in rats with FCA monoarthritis (50 µg) of one hind paw ankle joint. 2-way ANOVA was performed. Data shown as mean ± SEM, n = 25 per group. (Adapted from **Paper IV**, Fig 5A)

In mice, significant effects on stationary guarding index (DWB) was achieved one day after MEDI578 injection for the two lower doses (0.3 mg/kg and 1.0 mg/kg) and at three days for all doses. When the same mice were tested in the CatWalk™, a significant reduction of guarding index during locomotion was attained only with the high dose (3.0 mg/kg) three days after injection, and the dose relationship was weak (Figure 12).

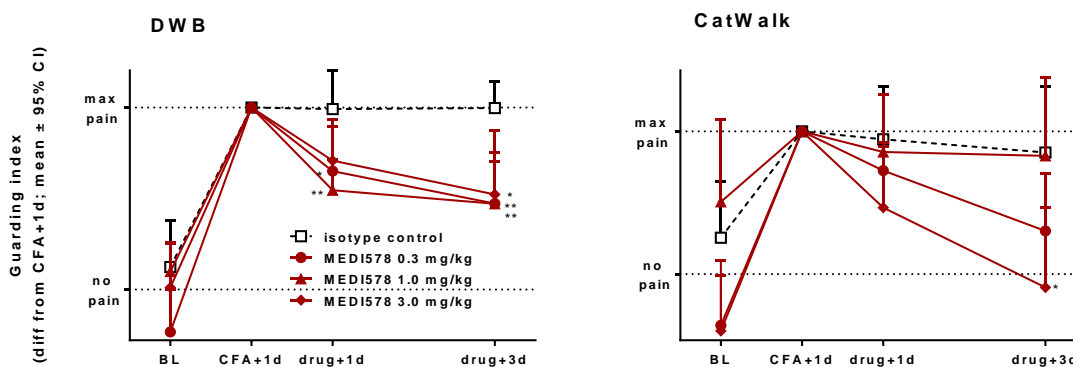


Figure 12. Guarding index before and after one single subcutaneous injection of MEDI-578, assessed on the DWB and CatWalk in mice with CFA monoarthritis (10 µg) of one hind paw ankle joint. The guarding index of naïve mice before CFA injection is referred to as “no pain”, while “max pain” refers to results at one day after CFA injection, before drug treatment. Each individual mouse’s difference between measurements at “max pain” and the corresponding measurement at each time point after treatment start was used for subsequent analysis. 1-way ANOVA followed by Dunnett’s test versus isotype control mice was performed. Data shown as mean ± 95% CI, n = 11 per group. (From **Paper VII**, Fig 4 A and B)

Willingness to place the injured paw on the floor was increased already one day after treatment by some doses, and all doses reached significant effects three days after injection, both when assessed when the mice were stationary and during locomotion (**Paper VII**, Figs. 4 C and D).

All data from the drug effect studies with mice were pooled and results for each individual mouse from the DWB versus results from the CatWalk™ were plotted. Stationary guarding index (DWB) and guarding index during locomotion (CatWalk™) correlated strongly, as did duration of floor contact versus duty cycle (**Paper VII**, Figs 4 E and F; $p < 0.0001$ for both).

4.4 MICE WITH NON-FUNCTIONAL NGF ARE PROTECTED FROM ARTHRITIS-INDUCED CHANGES IN GAIT AND WEIGHT BEARING

Paper VIII

We characterized mice with mutations in the gene coding for NGF. Homozygous mice with the human NGF (hWT mice) or with the human NGF containing a mutation, replacing the arginine 100 by glutamic acid in the NGF β gene (hR100E mice), were tested with respect to their response to basic sensory tests and their response to acute and longer lasting models of inflammatory pain. In addition, nervous tissue from skin, the sciatic nerve and DRG were analysed. No difference was found between the two with respect to development of the nociceptive system (Figure 13).

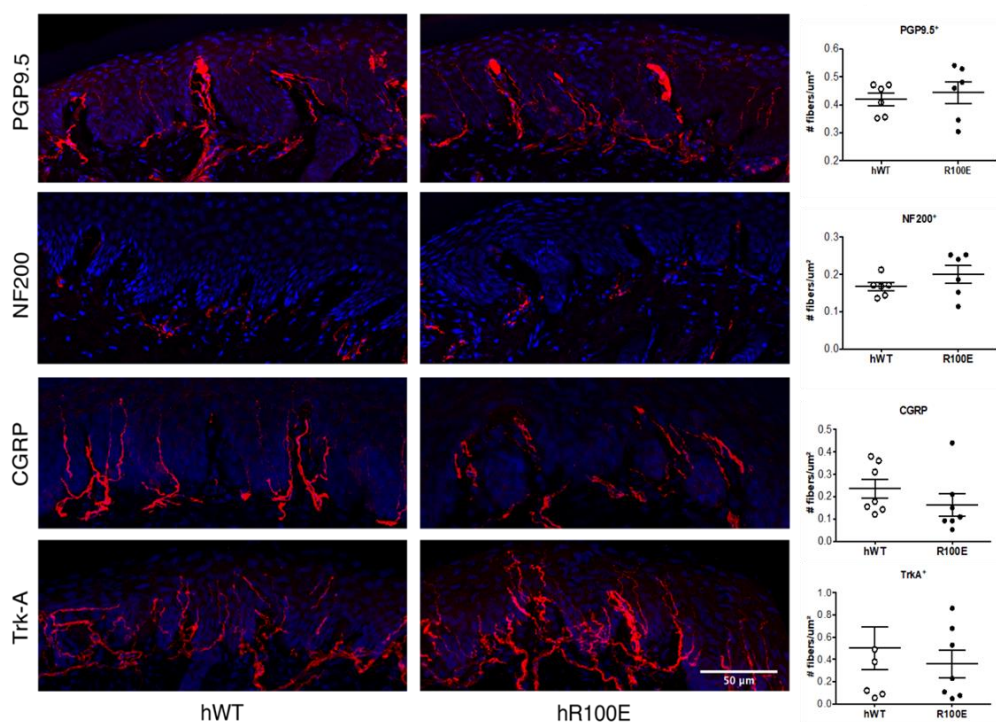


Figure 13. Representative images of PGP9.5(+), NF200(+), CGRP(+) and TrkA(+) nerve fibres in the glabrous skin of hWT (left) and hR100E (right) mice. Fibre area and length from the contralateral hind paw skin of hWT (white; $n = 7$) and hR100E (black; $n = 7$) mice. To compare levels between groups, t-test was performed. Data presented as mean \pm SEM. (From **Paper VIII**, Fig 2 E and F)

Neither was any difference between hWT and hR100E mice regarding baseline mechanical, cold or thermal sensitivity found (**Paper VIII**, Fig 3), nor in response to acute pain as measured with the formalin test (**Paper VIII**, Fig 4). Further, when testing mechanical and thermal hypersensitivity after ankle joint injection of CFA (5 μ l of 10 mg/ml), no significant difference was seen between the two (**Paper VIII**, Fig 5 B and C). However, when assessing stationary

weight bearing (Incapacitance tester) and gait and weight bearing during locomotion (CatWalk) after knee joint injection of CFA in those mice, we did see a significant difference (Figure 14).

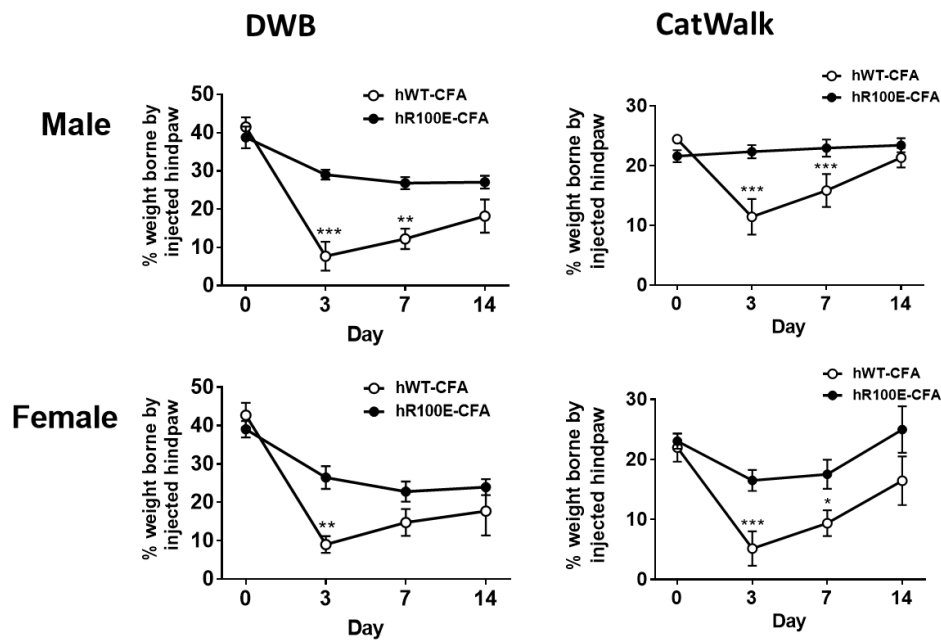


Figure 14. Stationary (dynamic) weight bearing and weight bearing during locomotion assessed on the DWB and CatWalk respectively, before and after intra-articular knee joint injection of CFA (100 μ g) in hWT and hR100E mice of both genders. 2-way ANOVA followed by Bonferroni's test versus baseline values before CFA injection was performed. Data shown as mean \pm SEM. The number of animals were: male; hWT n = 7, hR100E n = 7; female; hWT n = 5, hR100E n = 7 per group. (Adapted from **Paper VIII**, Fig 6 C and 7 C)

5 DISCUSSION AND CONCLUDING REMARKS

The aim of this thesis has been to evaluate models and methods for joint inflammation and injury, in order to find those that reflect the symptoms that bring human patients to seek medical help; pain while walking. The work for this thesis spans over many years, with the first studies being conducted in 1999 in Utrecht on the CatWalk. The pharmacological focus on NGF and TRPV1 stems from direct experience of working in projects aiming to develop pain relieving drugs using these molecular targets. It can be argued that it's common knowledge that treatments affecting the NGF pathway works and that our work thus does not bring new insight. However, at the time when we first tested the anti-NGF antibody in 2005, knowledge of its preclinical or clinical effect on joint pain were not widespread.

Our first aim was to investigate three models in rats where an injection into the joint caused pain-like behaviour; carrageenan, CFA and MIA. The two first mainly cause robust inflammation, while MIA destroys the cartilage. In mice we also used intra-articular injection of CFA, and one surgical model; ACLT. All models showed reduced weight bearing, albeit to differing extent, of the injured paw. However weight bearing can be assessed in many different ways, and we have tested a number of those.

First, to measure static weight bearing in the rat models we used the Incapacitance tester, where the animals were put onto a test device with each hind paw resting on a scale, giving the weight exerted by each paw. As the front paws rested on a ramp, without contributing to the total weight, the weight bearing for both hind paws always amounted to 100 percent of the body weight. Animals are constrained in a holder and have to be trained to get reliable results. It can be challenging to get each hind paw to rest on the right scale, and it's difficult to ascertain that the animals are balanced in the middle without their body leaning on the walls. Even so, it quickly gives values of weight bearing. In a previously published alternative method, rats were put in a box with a flat floor containing force-plates where the two hind paws are placed to give the percentage of weight bearing for each, again amounting to a total of 100% for the hind paws (Schött et al., 1994).

Second, the DWB device was used to assess stationary weight bearing in the mouse models we tested, to get results from all paws. Here, the animals could move freely and no training was necessary. The floor was equipped with a sensor mat where the weight of each paw was registered indirectly at periods of immobility, but analysis became very time consuming when the necessary validation of the signals from the sensor mat was compared to the videotape simultaneously recorded from above.

The third approach, assessing weight bearing and gait during locomotion, has been the one mostly used for our studies. Apart from only getting information on hind paw weight bearing, both front paws are also measured. This gives added knowledge on how animals choose to compensate for the reduced weight put on an inflamed or injured hind leg. One or two training sessions are needed, but after that it takes only a few seconds for an animal to cross the walkway, and the quick validation of the automated analysis rapidly gives the final results. This approach also gave many other parameters pertaining to gait, such as the relative time spent

putting weight on the floor by each paw, the interlimb coordination, i.e. the regularity of the four paw's placement on the floor while walking, and distance between the paw placements. Furthermore, we tested an approach where mice were forced to walk within a restrained area on a transparent floor (TreadScan from CleverSys); a belt that moved and forced the mice forward to avoid getting pushed from behind or even get squeezed between the bumper at the end of the walkway and the floor (not reported). Mice induced with CFA monoarthritis became very stressed by the setup and many refused to move. The basis for calculating stance pressure (\approx weight bearing) was the colour of the plantar side of the paw, which is calibrated for each strain of mice. The assumption that the skin colour is changed as it's placed on the floor and small vessels get occluded does not seem to hold true.

All three ways of getting data that we decided to continue with; static, stationary and during locomotion, show that joint inflammation alters weight bearing. For the stationary weight bearing and that during locomotion we introduced a new calculated result parameter; the guarding index, which shows the shift of body weight from the injured hind leg to the non-injured leg. Guarding index is the parameter mainly used in the figures of this thesis. The models we have used give varying effects, both in terms of magnitude and of duration. Thus, the two mainly inflammatory models (carrageenan and CFA) showed the largest difference between those with injection and controls, whereas the MIA model showed an intermediate difference and the surgical ACLT had difficulties reaching significant difference compared to controls. Unfortunately, this is opposite to what would be advantageous when studying OA.

Another aim was to test whether conventional pain relieving drugs could normalize the effects on gait and weight bearing in the models we used. The carrageenan model, which is the model with shortest duration, provided a large difference in guarding index between animals with monoarthritis and controls and is therefore suited for pharmacological tests. NSAIDs were able to completely restore weight bearing, reducing the guarding index to levels below that of controls, but opioids did not reach a similar efficacy. These drugs also showed effect on gait parameters such as fraction of total step duration (duty factor) and regularity index. Thus it seems that this model lends itself primarily to testing drugs that inhibit COX enzymes. In contrast, the NSAIDs tested in the longer-lasting CFA monoarthritis model were unable to fully normalize the weight bearing decrease, reaching only around 40% reduction of the guarding index. The smaller differences between the two hind leg's weight bearing (guarding index) in the MIA and the ACLT models made it almost impossible to test pain relieving drugs with the number of animals per group conventionally used in those studies. It's unfortunate that models mimicking the structural changes seen in OA don't give the robust behavioural changes needed for pharmacological testing of pain-relieving drugs. Perhaps several models could be used to include the different aspects of OA pain, in such a way that the CFA-induced monoarthritis, albeit definitely not a model of OA, could represent pain occurring from the inflammatory aspect, whereas the MIA model more closely resembles the phase of OA pain when the cartilage becomes degraded. These two models cannot, however, be used to understand the underlying mechanisms of progression disease. To more closely look at those aspects the

surgical and natural occurring models are better suited. It's a challenge to find a model that both mirrors the structural changes in OA, and that makes it possible to test new chemical targets for pain treatment. The work on the models in this thesis can be summarized in table 3.

Table 3 Comparison between the models used

Model	Inflammation	OA-like structural changes	Magnitude of weight bearing changes	Affected by conventional pain drugs
Carra-geenan	+++		+++	+++
CFA	+++	+	+++	++
MIA	+	++	++	+
ACLT		+++	+	

The next aim of this thesis was to explore the role of NGF and the ion channel TRPV1 in arthritis-induced spontaneous pain. For this we used pharmacological treatments developed by AstraZeneca; one anti-NGF antibody and two small molecule inhibitors of the Trk receptors A, B and C. In the carrageenan monoarthritis model the pan-Trk inhibiting compounds showed significant effects, but the efficacy was below that of all NSAIDs and opioids tested. However, in the CFA-induced monoarthritis the anti NGF antibody and the pan-Trk inhibitors were able to normalize the CFA-induced increase of guarding index with 60-80%, thus showing a stronger effect than that by NSAIDs in this model. Thus it's clear that the models engage different parts of the immunological system, and that the CFA-induced model recruits other pathways than those entirely dependent on COX.

The genetically modified mice given to us by AstraZeneca, where the arginine at R100 was replaced by glutamic acid in the NGF β gene, responded normally when testing for baseline sensitivity and effects of formalin-induced nociception, and showed normal response to tester-evoked sensory stimuli after induction of CFA-induced monoarthritis. This was in line with the normal development and differentiation of the nociceptive system found in nervous tissue of these mice. In contrast, assessment of gait and weight bearing in the NGF R100E mutated mice showed a significant lack of pain-like response compared to wild-type mice, pointing to the larger susceptibility by this mutation in deep tissues of the joint.

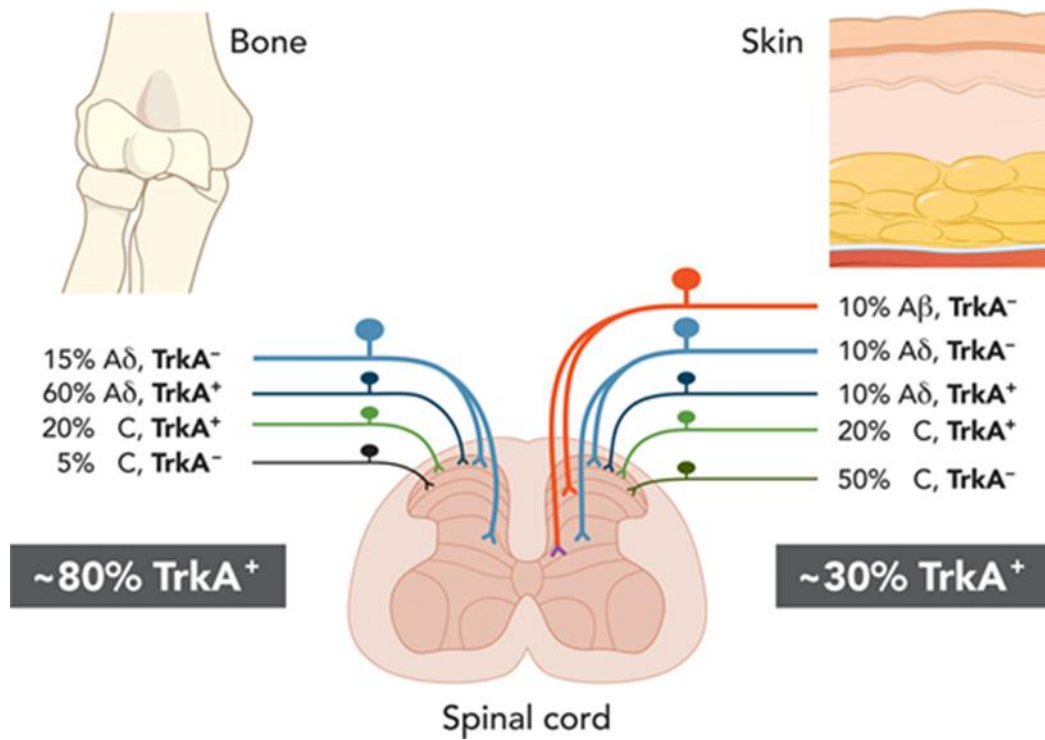


Figure 15. The difference in percentage of TrkA positive nerves in bone compared to that found in skin. (Reprinted with permission from Mantyh et al., 2011)

One of the possible reasons for this disparity between skin and joint sensitivity could be the different expression of the TrkA receptor; only about 30% of the sensory nerves in skin are TrkA positive, while more than 80% of the sensory nerves in bone are TrkA positive (Figure 15; Mantyh et al., 2011; Guedon et al., 2016). The information gained by using gait and stationary weight bearing or weight bearing during locomotion would not have been discovered with the traditional tester-evoked assessments.

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